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The Chemistry of Nucleic Acids

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PREFACE

OUR present knowledge of the structure and function of the nucleic acids has accumulated from studies involving a wide variety of different disciplines, ranging from physics through chemistry and biochemistry to biology and genetics. A comprehensive work covering all aspects of the structure and function of nucleic acids would require several volumes and would be beyond the compass of any one author. I have therefore concentrated, in this book, on the chemistry and structure of the nucleic acids. The word structure is used here in its widest sense, thus in addition to the detailed architecture of the nucleic acid molecule giving the relative positions of the atoms, the nature of the bonds, and the size and shape of the macromolecule, the heterogeneity of nucleic acid preparations brought about by different sequences of nucleotides, by different nucleotide composition, and different molecular size is also discussed. Such an interpretation is clearly necessary, since if the nucleic acids carry the genetic code, as is currently believed, and also possess other biological functions, the structure must be all important in determining these functions.

My interest in the chemistry of the nucleic acids was first aroused by the late Professor J. M. Gulland with whom I was privileged to work for several years. I wish to record my appreciation of his guidance and encouragement when our ideas of the macromolecular structure of nucleic acids were first taking shape.

I wish to record my thanks to Miss Shea Smith for considerable assistance throughout the preparation of the manuscript, for typing the manuscript, and for help with the preparation of the indexes. I also wish to thank my colleagues, Drs. J. H. Coates, T. Kuruscev, and R. B. Inman for many helpful discussions.

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1

INTRODUCTION

THE combination of chemical, cytological and genetical studies has led to the realization that nucleic acids are of fundamental importance in controlling the metabolism, reproduction and growth of living systems. Almost all metabolic processes function through enzymes whose synthesis is controlled by the genes, the units of heredity. The gene must be capable of replication during cell division and it must also be able to control the synthesis of a specific protein molecule. It has been known for some time that the chromosomes, which are the cellular structures incorporating the genes, consist of nucleic acids and proteins, but convincing evidence has only recently been given that the genetically significant part of the gene is composed of nucleic acid and not protein.

The idea that it is the nucleic acid which carries the genetic information stems from the discovery by Avery, Macleod and McCarty[1] that the ' transforming principle,' first described by Griffith[2], which under appropriate conditions will induce the transformation of unencapsulated R variants of pneumococcus type II into fully encapsulated S cells of type III, is a highly polymerized deoxypentose nucleic acid[3]. This work did not immediately receive the widespread recognition that it deserved, largely because ideas concerning the structure of nucleic acids were still prejudiced, at that time, in favour of the erroneous tetranucleotide structure. However, with the appreciation of the macromolecular character of nucleic acids and in particular through the work of Hershey and Chase[4] concerning the nature and action of phages, the implications of the work of Avery and his collaborators have become widely accepted. Hershey and Chase[4] were able to demonstrate that when coliphage is added to *Escherichia coli* cells in a suitable environment, the nucleoprotein constituting the phage dissociates, the deoxypentose nucleic acid and a small amount of soluble protein enter the cell while most of the protein component remains outside. In the subsequent stages of phage reproduction occurring in the host cell, specific protein and deoxypentose nucleic acid are synthesized which are different from any of the components of the non-infected host. Such species-specific materials, it is generally assumed, are produced under the influence of specific genetic determinants, in this case either the deoxypentose nucleic acid or the soluble protein entering the cell or both. Hershey[5] has shown that the material which enters the cell, what he calls the 'germinal substance,' is 97 per cent deoxypentose nucleic acid and 3 per cent protein and that a decisive assignment of the phage genetic functions to one component in preference to the other cannot be made at present. However, Herriott[6] considers that in view of the nature of the bacterial transforming factors and from structural considerations, the deoxypentose nucleic acid appears the more attractive choice. This view is substantiated by the study of the rescue of genetic markers from ultra-violet-inactivated phage by Doermann, Chase and Stahl[7] and the isotopic tracer studies of Stent[8]. Further evidence for the unique part played by nucleic acids in biological processes comes from the observation, obtained independently by Fraenkel-Conrat[9] and by Gierer and Schramm[10], that the pentose nucleic acid isolated from tobacco mosaic virus possesses virus activity which can account for almost all the activity of either the native virus or the reconstituted virus formed by the recombination of the dissociated nucleic acid and protein.

From studies such as these, it has proved evident that the once held view that the primary coding of genetic information resided in the protein portion of the chromosomes must be abandoned, and it is now generally accepted that the primary genetic material is deoxypentose nucleic acid, except in the plant viruses, where pentose nucleic acid takes its place[11].

In order that the nucleic acids may convey genetic information, a pattern or code must be incorporated in their chemical structure. The nucleic acids are co-polymers in which the monomers, known as nucleotides, consist of a purine or pyrimidine linked to a sugar phosphate. In any one nucleic acid there is only one kind of sugar, but generally four different purines or pyrimidines. Although there is no direct information concerning the sequence of purines and pyrimidines, the fact that they are the only variable constituents suggests that they are arranged in a definite sequence and that this sequence constitutes the genetic code. The length of the nucleic acid molecule varies and is dependent not only on the particular type of tissue extracted, but preparations from a given tissue are invariably heterogeneous, as shown by a wide distribution in the sedimentation coefficient[12,13]. This heterogeneity may, in part, be produced artificially during extraction, but in view of the observation that the fractionation of nucleic acids by anion exchangers[14,15] shows a correlation between the composition of the eluting solution and the sedimentation coefficient, and that the sedimentation coefficient distribution within the fractions is much narrower, the possibility arises that the nucleic acids of different genes are of different lengths. Thus in addition to the genetic code being associated with the sequence of purines and pyrimidines, it may also be determined by the length of the nucleic acid molecules.

The study of nucleic acids and in particular the study of their biological function, has been considerably stimulated in recent years by the proposal in 1953 by Watson and Crick[16] of a structural model for deoxypentose nucleic acid which, with one or two minor modifications, is now widely accepted as the basic structure for this type of nucleic acid. This structure, which is fully discussed in later chapters, consists of two polynucleotide chains, which run in opposite directions, are coiled round each other to form a double helix, and are bound to each other by specific hydrogen bonds between the purines and pyrimidines. The complementary arrangement of the purines and pyrimidines of the two polynucleotide chains produced in this way suggested to Watson and Crick[17] that the nucleic acid molecule replicates itself directly by having each chain serve as a template for the formation of its complement. This stimulating suggestion presents many problems involving the separation of the intertwined polynucleotide chains, and several modifications of the replication procedure have been suggested which have been fully discussed by Delbrück and Stent[18]. Although modifications have been made to both the original structure and replication mechanism suggested by Watson and Crick, their contribution, based, as it was, on the accumulated chemical and physical evidence from several laboratories, represents an important landmark in the development of our knowledge of nucleic acids.

The discovery of the nucleic acids was the result of the work of Miescher, who, working in the laboratory of Hoppe-Seyler in Tübingen in 1868–69, isolated from the nuclei of pus cells a substance which he called ' nuclein.' This substance, which was acidic in nature, warranted special interest as it contained a relatively high proportion of phosphorus and at that time lecithin was the only known organic compound in tissue which contained phosphorus. Miescher submitted an account of his work to Hoppe-Seyler who found it so interesting and surprising that he repeated, personally, the experiments of Miescher on pus cells and engaged two of his students, Plósz and Lübavin to test the methods of Miescher on materials other than pus (*viz.* egg yolk, yeast, casein and the red cells of birds and reptiles). Having confirmed the discovery of ' nuclein ' the work of Miescher was published along with that of Hoppe-Seyler and his pupils in Hoppe-Seyler's *Medicinisch-chemische Untersuchungen* in 1871[19]. A convenient and general method of preparation of ' nuclein ' was later described by Altmann[20], to whom also must be credited the introduction of the term ' nucleic acid.'

The work of Miescher, which amply demonstrated the highly polymeric character of nucleic acid, was unfortunately rather overlooked by later workers, who, for investigations using the degradative methods of organic chemistry, made use of material which had been extracted from tissue by methods involving heat treatment and the use of acid and alkali. Such methods of extraction yielded a product which, while no doubt suitable for the degradative studies and the examination of hydrolysis products, bore little resemblance to the native nucleic acid.

Many assumptions based on investigations making use of degraded material led to erroneous conclusions, some of which may have seriously retarded the development of ideas on the structure and function of nucleic acids. For example, the conception that the nucleic acid molecule was a tetranucleotide, which was accepted for some ten to fifteen years, grew up through the discovery of four nucleotides in approximately equimolecular proportions in the hydrolysate of yeast nucleic acid and a determination of molecular weight[21], later shown to be erroneous[22] which indicated a particle size corresponding to a molecule composed of four nucleotides. In spite of this criticism, however, the degradative studies by the classical methods of organic chemistry were of fundamental importance in determining the elementary molecules which together constitute the nucleic acids.

From this work there emerged the idea of the existence of two distinct types of nucleic acids. One, the nucleic acid from yeast, on hydrolysis yields adenine, guanine, cytosine, uracil, phosphoric acid and a sugar recognized by Hammarsten as a pentose and identified by Levene and Jacobs[23] as ribose. The other, the nucleic acid from thymus gland, yields adenine, guanine, cytosine, thymine (5-methyl uracil), phosphoric acid and a sugar shown by Levene and London[24] to be a deoxypentose and identified by Levene, Mikeska and Mori[25] as deoxyribose. These two nucleic acids thus came to be called ribonucleic acid and deoxyribonucleic acid, respectively, and since most nucleic acids of animal origin appeared to resemble that from thymus while those of plant origin were similar to that from yeast, the idea became prevalent that the pentose nucleic acids were characteristic of plants while the deoxypentose nucleic acids were characteristic of animal tissues.

This classification, however, was never free from objection and from its inception exceptions were continually being discovered. Conclusive evidence of its invalidity came from the histochemical studies of Brachet[26], the ultra-violet spectrophotometric examination of tissues introduced by Caspersson[27] and the chemical analysis of cells by Davidson and Waymouth[28]. More precise analysis of the chemical constituents of cells and tissues became possible with the introduction of the methods of paper[29–31] and ion-exchange[32] chromatography. From studies such as these, it is generally concluded that the cytoplasmic components contain pentose nucleic acid, while the nuclei contain deoxypentose nucleic acid and small amounts of pentose nucleic acid. It has thus been demonstrated that both types of nucleic acid are present in all types of cell, whether of plant or animal origin and that the main biological distinction between pentose nucleic acid and deoxypentose nucleic acid is that the former is mainly found in the cytoplasm, while the latter is exclusively, or almost exclusively, found in the nucleus.

The identification of the sugar in the nucleic acids has only been rigorously determined in a few instances. The only sugars isolated from nucleic acids so far are D-ribose and 2-deoxy-D-ribose. The pentose sugar has been identified as D-ribose in the pentose nucleic acids of yeast[23] and liver[33], but has been shown to be chromatographically identical with D-ribose in pentose nucleic acids from a large number of sources[31]. The deoxypentose sugar in the deoxypentose nucleic acid of thymus has been identified as 2-deoxy-D-ribose[25], but again the sugars in deoxypentose nucleic acids from other sources have been shown to be identical with it by chromatographic methods[30]. Throughout this book the generic names ‘pentose nucleic acid’ and ‘deoxypentose nucleic acid’ will in general be used, except where reference is made to a specific nucleic acid of known composition.

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2

THE ISOLATION OF NUCLEIC ACIDS

INTRODUCTION

The aim of research in nucleic acids, in common with many biochemical studies, is to relate the chemical structure and reactivity of the isolated nucleic acids to their function in living organisms. The isolation of cell constituents of complex structure and high molecular weight must always raise the question as to whether the isolated preparation may be regarded as in any way equivalent to the original substance in the cell. Ideally, isolation procedures should be such as will produce a nucleic acid preparation which retains, in every detail, the structure existing in the original cell. However, nucleic acid has always been found associated with protein in the cell. Whether all of the nucleic acid in cells occurs as a nucleoprotein cannot at present be stated, but there can be little doubt that most, if not all, of the nucleic acid is present in the cell as a biologically active nucleoprotein. It is possible that nucleoproteins may be isolated more or less unchanged, but it is very difficult to distinguish between native nucleoprotein and artifacts such as protein nucleates, produced by the simultaneous isolation of protein and nucleic acid from cells. To separate the nucleic acid from the protein various procedures have been adopted, but whether these be mild or strong, the fact remains that if the nucleic acid occurs in the cell combined with protein, groups originally joined to protein will become free and the danger of secondary reactions may be considerable.

The isolation of nucleic acids is further complicated by our lack of knowledge of their exact functions in the cell. Unlike many of the proteins, such as enzymes and antibodies, nucleic acids possess no easily measurable biological activity. The criteria for the satisfactory isolation of a specimen of nucleic acid are therefore much more difficult to define than for the analogous case of the proteins and many arbitrary standards have from time to time been employed; these have included chemical analysis, molecular weight, ultra-violet absorption spectra, viscosity and acid–base titration. These criteria can strictly only be used for comparing preparations and make use of such not necessarily valid assumptions as, for example, the higher the molecular weight the better the preparation. It is to be emphasized, therefore, that at present there is no absolute criterion of a satisfactory preparation of nucleic acid such as exists for some proteins and viruses.

The first preparations of nucleic acids described by Miescher[1], Altmann[2], and Kossel and Neumann[3] were devised on the assumption that nucleic acids were labile substances and that reactive reagents and heat would produce fundamental changes in the nucleic acid. This assumption is now known to have been correct and all modern methods of preparation employ the mildest possible conditions. However, in 1899, Neumann[4] described a modification of the original Altmann procedure which involved the heating of the minced organs in a 3 per cent solution of sodium hydroxide, and which was to set the pattern for preparations for the next thirty years[5]. Thus Levene and Bass[6] state ' the old traditional fear of using energetic methods for the separation of the nucleic acid from the protein was abandoned '.

This development probably led to an acceleration of the study of the products of the hydrolysis of nucleic acids by the classical methods of organic chemistry, but nevertheless was also responsible for the acceptance of a very erroneous value for the molecular weight of the nucleic acids. Thus while it appears very probable that both Miescher and Kossel realized the complex nature and macromolecular structure of nucleic acids, the later workers considered nucleic acids to be much smaller. This view was not discredited and the true macromolecular character of nucleic acids again realized until a return was made to the mild methods of preparation.

Nucleic acids occur in the cell in close association with protein and since the most difficult step in the preparation of nucleic acids is the separation of the nucleic acid from the protein, the isolation is now generally carried out in two stages: firstly, the isolation of the nucleoprotein, which may or may not be isolated in the solid state, and secondly the separation of the nucleic acid from the protein. In this procedure the separation of the pentose from the deoxypentose nucleic acid is carried out while both acids are combined with protein by making use of the marked difference in the solubility of pentose and deoxypentose nucleoproteins in electrolyte solutions, described by Mirsky and Pollister[7]. Procedures which have been described for the separation of pentose and deoxypentose nucleic acids[8] after separation from the protein, and which employ strong reagents, are not satisfactory.

THE ISOLATION OF DEOXYPENTOSE NUCLEOPROTEIN

Excluding those methods which involve the use of acid or alkali, two main methods are used for the isolation of the nucleoprotamines and nucleohistones. The difference between these methods lies in the ionic strength of the solution used for the extraction of the nucleoprotein from cell tissue; the first method employs solutions of water or of low ionic strength[9–28]; the second method uses solutions of high ionic strength[7, 29–39]. For the purpose of isolating a sample of deoxypentose nucleic acid, there is probably little to choose between the two procedures, but for a preparation of nucleoprotein the former method is to be preferred since there exists considerable evidence that in strong solutions (1M) of sodium chloride a dissociation of the nucleic acid and protein takes place[7, 12, 26, 36, 37, 40–42] with, on precipitation, the formation of a protein nucleate which is unlikely to resemble in specific detail the original nucleoprotein.

The remarkable variation of solubility of deoxypentose nucleoprotein in solutions of electrolytes[7] enables a complete separation of this nucleoprotein from pentose nucleoprotein to be carried out. The solubility of deoxypentose nucleoprotein in solutions of sodium chloride of different concentration has been fully investigated by Frick[36] who showed that the solubility in 0·14M sodium chloride solution (physiological saline) was one hundredth of the solubility in pure water, while the solubility in 1M sodium chloride was at least twice that in water. The solubility of deoxypentose nucleoprotein thus shows a minimum in solutions of 0·14M sodium chloride. On the other hand, the pentose nucleoproteins are more generally soluble and in particular are soluble in 0·14M sodium chloride. Thus by washing minced tissue with 0·14M sodium chloride solution a separation of the deoxypentose from the pentose nucleoprotein may be effected.

During the isolation of the nucleoprotein from the cell, enzymic degradation must be prevented, or, if this is not possible, kept at a negligible level. Specific depolymerizing enzymes for nucleic acids appear to be present in most cells and during the isolation procedure the enzyme and the nucleoprotein are free in the same solution and the possibility of reaction exists. To reduce or prevent enzymic action a variety of precautions have been recommended. All preparations are made at 0°C or lower to reduce the rate of enzymic action and generally enzyme poisons such as arsenate[18], citrate[34], fluoride[33] or a chelating agent such as sodium ethylenediamine tetra-acetate are added to further suppress enzymic action.

Emphasis has already been laid on the necessity, during the isolation of nucleoproteins and nucleic acids, of avoiding the use of heat and of strong chemical reagents which will alter the pH of the solutions significantly from neutrality. Also, as first pointed out by Chargaff[43], the use of high speed mincers is to be avoided. There is now little doubt that the excessive use of such mincers (blendors or homogenizers) during preparation leads to degradation of the macromolecules. This is probably brought about either by local heating or ultrasonic cavitation effects. However, the use of such a mincer appears to be necessary in order to disrupt the cells, but it is essential that it should be used for as short a period as possible.

Two typical preparations, the first using solutions of low ionic strength and the second solutions of high ionic strength are given below.

Preparation of calf thymus nucleohistone using solutions of low ionic strength[26]

Calf thymus gland from freshly slaughtered animals is trimmed to remove fat, chilled immediately and processed without delay. All subsequent operations should be performed in a cold room at 4–6°C. Fifty-gramme portions of gland are triturated for 30 seconds in a high speed mixer equipped with cutting blades, with 50 ml. of an ice-cold mixture of 0·1M sodium chloride and 0·05M sodium citrate previously adjusted to pH 7. The supernatant fluid resulting from centrifugation at 2000*g* for 30 minutes is discarded and the sediment resuspended in 100 ml. of the sodium chloride–citrate solution and again centrifuged. This washing procedure may be repeated.

The sediment is then washed three times by thorough resuspension and centrifugation in order to remove electrolytes, using each time 50 ml. of distilled water, previously adjusted to pH 7 by the addition of sodium bicarbonate solution. During the final washing the sediment should swell, but not appreciably dissolve in the supernatant fluid. The gelatinous sediment is then blended for 15 seconds in a high speed mixer with 250 ml. of distilled water (adjusted to pH 7) and shaken overnight. A very viscous solution should result which after briefly stirring in the high speed mixer, is centrifuged for 30 minutes at 2000*g*. The nucleoprotein is precipitated by making the aqueous solution 0·15M with respect to sodium chloride by adding the calculated amount of a solution (approximately 0·2M) of sodium chloride. The precipitate is collected after 30 minutes by centrifugation and washed in the centrifuge with 0·15M sodium chloride and then, very briefly, with a small amount of distilled water.

Preparation of calf thymus nucleohistone using solutions of high ionic strength[7, 37]

Calf thymus gland is obtained as described above and all subsequent operations should be performed in a cold room at 4–6°C.

Fifty-gramme portions are blended in a high speed mixer equipped with cutting blades for 30 seconds, with 50 ml. of an ice-cold mixture of 0·1M sodium chloride and 0·05M sodium citrate previously adjusted to pH 7. The supernatant fluid resulting from centrifugation at 2000g for 30 minutes is discarded and the sediment resuspended in 100 ml. of the sodium chloride–citrate solution and again centrifuged. This washing procedure is repeated.

The sediment is now suspended in 100 ml. of 0·15M sodium chloride solution and blended for 15 seconds in a high speed mixer. The concentration of sodium chloride is then raised to 5 per cent by the addition of a 10 per cent solution. The viscous solution obtained after 24 hours is filtered through a coarse filter and the nucleoprotein precipitated by dilution with water to give an 0·15M solution. The precipitate is collected by centrifugation for 30 minutes, redissolved in 100 ml. of 5 per cent sodium chloride by blending in the high speed mixer for 15 seconds and again precipitated by diluting the solution to 0·15M. The precipitate is collected by centrifugation and washed, very briefly, with a small amount of distilled water. If the material is required dry, it is best dried from the frozen state.

The above methods of preparation, or modifications of them have been used for the preparation of deoxypentose nucleoproteins from a variety of tissues. Different techniques have been described[44–47], but do not appear to offer any particular advantage over the more common procedures. Certain nucleoproteins, however, notably those from some bacteria, require different procedures, such as extraction by dilute buffer solutions[48].

The above procedures for the preparation of deoxypentose nucleoprotein have been criticized by Doty and Zubay[27] largely on the ground that the final solution of the nucleoprotein possesses a gel-like character and is incompletely dispersed[24]. They consider that the irreversible formation of the gel is caused by (*1*) maintaining the nucleoprotein at concentrations above 0·08 per cent, (*2*) precipitating the nucleoprotein with 0·15M sodium chloride or alcohol or (*3*) allowing the salt-washed sediment to stand too long before homogenizing in water and by carrying out this homogenization too slowly. The following procedure, which yields a solution of the nucleoprotein in 0·0007M potassium phosphate buffer at pH 6·8 is notable for the absence of precipitation of nucleoprotein.

Preparation of calf thymus nucleohistone by the method of Doty and Zubay[27]

Working in a cold room, 20 g of fresh, frozen thymus gland are chopped and homogenized in a high speed mincer (this is run for 1 minute at half speed and for 3 minutes at a very low speed just sufficient to agitate the mixture) with 200 ml. of a solution containing

0·075M sodium chloride and 0·24M sodium ethylenediamine tetra-acetate at pH 8 and a few drops of capryl alcohol. The homogenate is strained through cheesecloth and the filtrate is centrifuged at 2000 rev/min for 10 minutes. The sediment is again homogenized in the high speed mincer (at slow speed only, for 3 minutes) in the same volume. The homogenate is centrifuged as before and the supernatant decanted. This washing procedure is repeated six more times. The final sediment is dissolved by rapid mixing with water in the mincer starting with 50 ml. of water and increasing this to 1 l. in a few seconds. The mixture is quickly transferred to a beaker and stirred for a hour and then dialysed against 0·0007M potassium phosphate buffer at pH 6·8.

Doty and Zubay consider, largely on the basis of particle size determination and N/P ratios, that this preparation may have yielded the principal structural units of the chromosomes and that the nucleoprotein has not therefore been changed in any way from its original ' native ' condition. However, this procedure has recently been criticized by Dounce and O'Connell[28] who consider that the formation of a gel by nucleoprotein solutions is not to be avoided, but is regarded by them as an indication of the presence of intact deoxypentose nucleoprotein, where the deoxypentose nucleic acid is attached to the residual lipoprotein, most probably through covalent bonds[49]. They thus consider that the method of preparation employed by Doty and Zubay produces a complex material consisting of several components and not unlike the isolated chromosomes first obtained by Mirsky and Pollister[7] and Mirsky and Ris[50]. They conclude that the claim of Doty and Zubay that the procedure yields a fundamental unit of chromosomal structure lacks experimental evidence.

THE ISOLATION OF THE SODIUM SALT OF DEOXYPENTOSE NUCLEIC ACID

In order to isolate a macromolecular sample of deoxypentose nucleic acid, all solutions used throughout the preparation must have a pH value close to neutrality. It therefore follows that the sodium salt is isolated, not the free acid. This fact is now generally recognized although the preparations are frequently referred to, somewhat inaccurately, as preparations of nucleic acid. The material finally isolated should be free from proteins, polysaccharides, lipids, *etc.*, pentose nucleic acids and inorganic salts. In general the procedure adopted should employ only mild reagents, and, as has been stated above, the use of heat, acid, alkali and the excessive use of high speed mincers are to be avoided. It should be emphasized that heating should not only be avoided during the preparation, but also when preparing solutions for subsequent physico-chemical study, since Peacocke[51] has shown that irreversible changes are produced by drying a sample in a vacuum at 110°C.

It has also become evident recently, largely as a result of the spectrophotometric studies of Thomas[52], that the reduction of the sodium chloride (or other inorganic neutral salt) concentration below 10^{-3}M, at low nucleate concentrations, results in irreversible changes in the structure. Thus the reduction of the sodium chloride content of deoxypentose nucleic acid solutions to concentrations below this value, by thorough dialysis, is to be avoided.

It is not possible to formulate, except in very general terms, the criteria of a satisfactory preparation. Thus while it is evident that a satisfactory preparation must have a high molecular weight and the solutions in water must have a high viscosity, the exact molecular weight and intrinsic viscosity required cannot be stated. However, one useful criterion, which has been used with success in several laboratories, has been suggested by Chargaff[53]; this is that the maximum absorption in the ultra-violet in solutions of deoxypentose nucleic acid containing sodium chloride (concentration $> 10^{-3}$M) and at pH 7, between 257 and 261 mμ, should have an $\epsilon(P)$ value of about 6600 (where $\epsilon(P)$ is the extinction coefficient per mole of phosphorus).

The normal procedure adopted for the isolation of deoxypentose nucleic acid from most tissues is to isolate first the nucleoprotein (or protein nucleate) either in the form of the dried solid, or better as a solution, by procedures similar to those outlined above and then to remove the protein and isolate the sodium salt of deoxypentose nucleic acid. Four main procedures have been introduced for the removal of the protein.

Hammarsten[40] developed the method, later improved by Signer and Schwander[54], in which, after isolating the nucleoprotein in solution, the protein is precipitated by saturating the solution with sodium chloride. The protein gradually precipitates over a period of about 4 days. This method has not been extensively used, possibly because of the long time required for the precipitation of the protein and of the difficulty of completely removing the last traces of protein.

Gulland, Jordan and Threlfall[35] used the method introduced by Sevag[55] and Sevag, Lackman and Smolens[15] for removing the soluble protein from any solution. This method involves shaking the solution with chloroform or carbon tetrachloride in the presence of a small amount of defrothing agent, such as amyl alcohol. This produces a stable gel, with the protein adsorbed at the water–chloroform interface. The gel is removed by centrifugation leaving a solution relatively richer in nucleic acid. Several such emulsifications are necessary to remove all the protein and while the method has several disadvantages, a protein-free preparation of deoxypentose nucleic acid is obtained.

The method now most frequently employed uses anionic detergents to dissociate the nucleoprotein complex and precipitate the protein. In 1938 Sreenivasaya and Pirie[56] first employed surface active materials to separate the nucleic acid and protein of tobacco mosaic virus, the anionic detergent used being sodium dodecyl sulphate. Later, in 1940, Bawden and Pirie[57] showed that sodium dodecyl sulphate, in the presence of alkali, readily separates the nucleic acid from the protein of several viruses. The method, however, was not developed into a preparative procedure for deoxypentose nucleic acids until 1951 when Marko and Butler[58] and Dounce, Simmons and Kay[59] described preparative procedures using sodium dodecyl sulphate. The latter authors later gave a detailed account of their method[60] which has since been widely used. Although sodium dodecyl sulphate has been most frequently employed, there is no reason to believe that its action is specific and other anionic detergents have been used with success[60, 61]. Generally the method yields a protein-free preparation although this is not always so[62]. Subsequent use of the chloroform-emulsification procedure, although not always desirable (see below) would ensure a protein-free preparation.

The last method which has been employed is that of Butler, Conway and James[63] which uses enzymic degradation to remove the protein. This method has not been used extensively outside Professor Butler's laboratory.

Details of the four procedures of general application are given below. The recommended procedure is that of Kay, Simmons and Dounce[60], employing anionic detergents to precipitate the protein, as experience has shown that this method gives a reproducible preparation of deoxypentose nucleic acid with little or no fractionation. Furthermore it has the advantage that the anionic detergent both dissociates the nucleoprotein complex into the nucleic acid and the protein and also precipitates the protein. With other procedures, for example the chloroform-gel method, it would appear that only dissociated protein is removed in the gel and that the presence of high concentrations of sodium chloride and possibly also the precipitation of the nucleoprotein complex by alcohol are necessary in order to bring about dissociation. Absence of the precipitation step leads to lower yields and to the presence of nucleic acid in the gel.

Extraction with anionic detergents[60]

Fifty grammes of fresh calf thymus are chopped into small fragments and homogenized in a high speed mincer (Waring blendor) for 1 minute (Kay, Simmons and Dounce[60] recommend 3 minutes, but the homogenizing time should be kept to a minimum to avoid degradation) with 200 ml. of ice-cold 0·9 per cent sodium chloride solution containing 0·01M sodium citrate. The homogenate is centrifuged at 0°C at 2500 rev/min for 30 minutes. The supernatant fluid is discarded and the sediment is washed by homogenization with the sodium chloride–citrate solution, followed by centrifugation three times. The sediment is then homogenized for 1 minute in a high speed mincer with 1 l. of ice-cold 0·9 per cent sodium chloride, and the material transferred to a large beaker and 90 ml. of a detergent solution (5 g of sodium dodecyl sulphate in 100 ml. of 45 per cent ethyl alcohol) added with stirring. The resulting gel is stirred vigorously at room temperature for 3 hours, during which it gradually becomes converted into a very viscous syrupy solution.

The sodium chloride concentration is then brought to 1M by the addition of 55 g of sodium chloride; this produces a marked drop in viscosity. The stirring is continued for 5 or 10 minutes to ensure complete solution of the sodium chloride. This concentration of sodium chloride should not be exceeded, since higher concentrations will cause precipitation of the detergent.

The solution is centrifuged at room temperature at 2500 rev/min (or at higher speeds which produce a clearer supernatant) to remove the protein sediment. Ethyl alcohol (95 per cent) is then added to the supernatant with stirring. A highly gelatinous precipitate forms which gradually loses water and develops into a white fibrous mass that can be wound up on the end of the stirring rod. The entrapped supernatant is pressed out and the precipitate washed three times with 95 per cent ethyl alcohol and then with acetone until the washings are no longer cloudy. The crude product is dried in air (yield about 2 g) and contains a small amount of protein impurity.

The crude product is suspended in 700 ml. of distilled water at room temperature and dissolved (taking about 2 hours) by rapid stirring. When solution is complete, 63 ml. of the detergent solution is added, followed by further stirring for 1 hour. The solution is then brought to a sodium chloride concentration of 1M by adding 45 g of sodium chloride, and centrifuged at 13,000 rev/min for at least 1 hour. The deoxypentose nucleate is precipitated from the supernatant by the addition of one volume of 95 per cent ethyl alcohol and washed as before. The product is again dissolved in 700 ml. of distilled water at room temperature by stirring for about 1 hour and 6·3 g of sodium chloride are added to bring the concentration to 0·9 per cent at which concentration any protein remaining in the preparation will precipitate, together with a very small amount of sodium deoxypentose nucleate. The solution is centrifuged at room temperature for 1 hour at 13,000 rev/min. The sodium chloride concentration in the supernatant is then increased to 1M by adding 35 g of sodium chloride and the sodium deoxypentose nucleate slowly precipitated by the careful addition with stirring of 95 per cent ethyl alcohol. The resulting fibrous product is washed as before with ethyl alcohol and then with acetone until the acetone washings are clear, and dried in air. (Yield about 1·3 to 1·4 g.)

An alternative precipitation procedure which may be used throughout this method is to blow a fine spray of the aqueous solution into 95 per cent or 100 per cent ethyl alcohol, by means of compressed air, and the precipitate collected by spooling as before. This method is particularly useful if for any reason, the deoxypentose nucleate is required to be precipitated from a pure water solution, as distinct from a 1M sodium chloride solution.

The following method was originally carried out by Gulland, Jordan and Threlfall[35] on a very large scale (commencing with 54·5 kg of calf thymus). Since preparations on this scale are unwise and generally not necessary, a smaller scale preparation, similar to that described by Chargaff[64], is given below.

Extraction with strong sodium chloride solution; removal of protein by the chloroform-gel method[35]

All operations are carried out in a cold room at 4°C. Two hundred and fifty grammes of fresh calf thymus are chopped and homogenized in a high speed mincer for 1 minute with 500 ml. of a solution containing 0·1M sodium chloride and 0·05M sodium citrate. The pH of this solution should be between 6·8 and 7·0. The sediment resulting from centrifugation at 2500 rev/min is washed twice more by homogenization with the sodium chloride–citrate solution. The tissues are now suspended and homogenized in 1250 ml. of 10 per cent sodium chloride solution, the viscosity of the solution markedly increases, and after extraction for 48 hours the solution is strained through cheesecloth and clarified in a Sharples centrifuge at 20,000 rev/min. The nucleoprotein complex is then precipitated by adding the clarified solution to 1·5 volumes of ethyl alcohol. The precipitate is either collected by centrifugation or by coiling on the stirring rod and washed with 70 per cent then 95 per cent ethyl alcohol and dried in a vacuum.

The nucleoprotein is suspended in 2 l. of 10 per cent sodium chloride by rapid stirring. The nucleoprotein is rarely completely soluble since some protein has been denatured by the precipitation procedure. An equal volume of a mixture of chloroform (three parts) and amyl alcohol (one part) is now added and homogenized in the high speed mincer for 30 seconds and centrifuged at 2500 rev/min. The supernatant is collected and the deproteinization procedure repeated, reducing the amount of chloroform–amyl alcohol each time until no gel appears at the water–chloroform interface. This generally takes eight to twelve separate operations. The supernatant is then added to two volumes of 95 per cent ethyl alcohol, the precipitate collected, drained and washed with 70 per cent, 90 per cent and 100 per cent ethyl alcohol and dried in a vacuum or in air.

Extraction with strong sodium chloride solution; removal of protein by saturation with sodium chloride[40, 54, 65]

Seven hundred and fifty grammes of calf thymus are chopped and homogenized in portions in a high speed mincer with 1·5 l. of 0·1M sodium chloride and 0·05M sodium citrate solution. The sediment resulting from centrifugation at 2500 rev/min is washed twice more by the same method. The tissues are now suspended in 2 l. of 10 per cent sodium chloride solution for 48 hours with occasional stirring. The solution is poured, with stirring, into ten times its volume of water and the precipitate separated by centrifuging. The nucleoprotein is then dissolved in 5 l. of 10 per cent sodium chloride, saturated with sodium chloride and the volume adjusted to 6 l. The solution is then kept for 12 to 14 days at 0°C.

A suspension of 500 g of Celite 545 in 1·5 l. of saturated sodium chloride solution is then added, the mixture stirred overnight and the precipitate filtered by suction with the aid of ‘ Hyflo-supercel ’ through a sintered glass filter (porosity No. 4). The filtrate is dialysed to reduce the sodium chloride concentration to about 1M and the sodium deoxypentose nucleate precipitated by adding the solution to two volumes of ethyl alcohol. The precipitate is collected, squeezed and washed with 70 per cent ethyl alcohol until free from chloride, then washed with 100 per cent ethyl alcohol and dried in a vacuum.

Alternatively the nucleoprotein may be precipitated a second time as a calcium nucleoprotein complex. After precipitating the nucleoprotein by dilution, as described above, it is homogenized in a high speed mincer with 1·8 l. of distilled water and left standing overnight at 0°C. The mixture is then centrifuged and 5 ml. of a 20 per cent solution of calcium chloride added to the supernatant solution with stirring. The precipitate is allowed to settle overnight and then collected by centrifugation, washed twice with 1 per cent calcium chloride solution and homogenized with 500 ml. of 10 per cent sodium chloride solution. This solution is then saturated with sodium chloride and added to 5 l. of saturated sodium chloride. After standing for 3 days at 0°C, the precipitated protein is filtered off with the aid of ‘ Hyflo-supercel ’ through a sintered glass filter (porosity No. 4) as before.

Extraction with strong sodium chloride solution; removal of protein by digestion with chymotrypsin[63]

Five hundred grammes of fresh calf thymus are frozen with solid carbon dioxide and minced. The tissue is suspended in about 3 l. of 0·14M sodium chloride solution and homogenized in a high speed mincer for 30 seconds and left standing overnight. The mixture is centrifuged at 2200 rev/min for 40 minutes and the supernatant discarded. The residue is resuspended in 1 l. of 0·14M sodium chloride solution, homogenized and centrifuged. This procedure is repeated twice, centrifuging for 30 minutes only.

The precipitate is homogenized in 1·5 l. of 0·14M sodium chloride, 1·5 l. of 20 per cent sodium chloride added with constant stirring and left overnight. The solution is adjusted to pH 7·4 to 7·8 and 200 mg of chymotrypsin added in two portions of 100 mg at 24 hour intervals. The solutions are left to stand at room temperature and filtered through cheesecloth. The sodium salt of deoxypentose nucleic acid is then precipitated by the addition of 3 l. of 70 per cent ethyl alcohol and washed with 70 per cent ethyl alcohol.

The precipitate is dissolved in about 3 l. of water and dialysed for a week against daily changes of distilled water at 5°C. If necessary the dialysate is filtered through cheesecloth to remove suspended matter and clarified by filtering several times in a Buchner funnel with successively finer grades of Celite. Forty ml. of saturated sodium chloride solution are added to each litre of clarified solution and the nucleate precipitated by adding an equal volume of 98 per cent ethyl alcohol. The fibrous precipitate is washed three times with 70 per cent ethyl alcohol, twice with 100 per cent alcohol and twice with acetone and dried in a vacuum over phosphorus pentoxide.

Miscellaneous procedures

While the methods described above may be applied for the extraction of sodium deoxypentose nucleate from a wide variety of mammalian tissues with little or no modification, special techniques have had to be devised for the isolation of the nucleate from some other sources, notably, micro-organisms, viruses, plant tissue and spermatozoa.

The first microbial deoxypentose nucleic acids to be isolated employing methods in which due attention was paid to avoiding degradation of the preparation, were those from pneumococci endowed with transforming activity[66]. They were isolated by extraction of the cells after lysis with sodium deoxycholate in the presence of sodium citrate, and deproteinized by the chloroform-gel method and finally precipitated as the calcium salt. A similar procedure[67] and isolation with anionic detergent[68] have been used for the isolation of the deoxypentose nucleic acid, also endowed with transforming activity from *Hemophilus influenzae*.

Although in these preparations the disintegration of the cell was achieved by mild lytic procedures, most micro-organisms require grinding with fine glass powder or treatment in a crushing mill as a necessary pre-requisite to extraction. Once the bacteria cells have been disrupted, extraction by one or other of the procedures described above or modifications thereof may be employed[48, 69, 70]. A satisfactory method for the isolation of deoxypentose nucleic acid from yeast cells has been made in this way[71].

Bacteriophages, especially the even-numbered T set, provide a useful source of deoxypentose nucleic acid ; this may be prepared[72] from the purified phage after precipitation in 95 per cent ethyl alcohol by resuspending in 0·2M sodium chloride and removing the denatured protein by emulsification with chloroform, using for the first three emulsifications pure chloroform and thereafter a 9 : 1 chloroform–octyl alcohol mixture. Apart from the phages, however, few isolations of deoxypentose nucleic acid have been made from the viruses.

The main difficulty in the extraction of deoxypentose nucleic acid from plant tissues is the contamination of the isolated material with pentose nucleic acid which, so far, appears to have been an insuperable difficulty in the way of the isolation of the pure acid. While the isolation of deoxypentose nucleic acids from fish and sea urchin spermatozoa and from testes may be made by following the standard procedures, their preparation from mammalian spermatozoa is unexpectedly difficult. Strong salt solutions fail to extract the nucleoproteins from mammalian spermatozoa unless some degradative pre-treatment is given. For example, a satisfactory preparation of a highly polymerized nucleic acid from human spermatozoa has been obtained only after treatment of the defatted spermatozoa with trypsin[73] (free of deoxyribonuclease). From bull spermatozoa, however, an initial extraction with potassium hydroxide, which must also degrade the nucleic acid, is required[74].

COMPARISON OF THE DIFFERENT METHODS OF PREPARING SODIUM DEOXYPENTOSE NUCLEATE

The only way that the products of the various methods of preparation can be properly compared is to make a systematic physicochemical study of the solution behaviour of the preparations. Such a complete study has not been carried out, but some information on the relative merits of the first three preparations is available.

Frick[75] has prepared specimens of calf thymus deoxypentose nucleate by the methods of Hammarsten, of Gulland, Jordan and Threlfall and of Kay, Simmons and Dounce. Unfortunately only qualitative studies were made on these preparations and the merits of the preparations discussed in terms of yield, but Frick nevertheless concluded that the Hammarsten procedure generally gives a preparation contaminated with protein. More useful and quantitative information comes from the light scattering and sedimentation studies of Doty and Bunce[76] and of Reichmann, Rice, Thomas and Doty[77] and the centrifugal fractionation studies of Shooter and Butler[78]. The chloroform-gel method gives a preparation having a lower molecular weight than either the Signer (Hammarsten) or the Kay, Simmons and Dounce procedures. It would appear, therefore, that some degradation occurs during the formation of the gel. The Signer and the Kay, Simmons and Dounce procedures give preparations of very similar molecular weight. The fractionation studies of Shooter and Butler have revealed that a marked fractionation occurs with the chloroform-gel method whereas the Kay, Simmons and Dounce procedure using an anionic detergent yields a deoxypentose nucleic acid with characteristics similar to those of the nucleoprotein.

It is thus to be concluded that at present the most satisfactory preparation procedure is that employing anionic detergents to separate the nucleic acid and protein as described by Kay, Simmons and Dounce, although it should be pointed out that the best x-ray diffraction patterns so far obtained were made using a Signer preparation.

The sodium deoxypentose nucleate, as prepared by the above methods, is obtained in a form which should be white and fibrous. The fibrous character of the precipitate has often been taken as a very rough indication of the satisfactory nature of the preparation, as degraded preparations are invariably powders. However a fibrous preparation can be converted into powder form[79] by repeated dissolution in distilled water followed by precipitation with ethyl alcohol. As the sodium chloride concentration is reduced, the nucleic acid becomes soluble in the alcohol and it may be precipitated in a powdery form by the careful addition of sodium chloride to a solution in isopropyl alcohol at 75°C. The product is still highly polymerized, although since it has been dissolved in water with a sodium chloride concentration of less than 10^{-3}M, some irreversible change of the type described by Thomas[52] may have occurred.

The most likely impurities in the sodium deoxypentose nucleate prepared by the above methods will be pentose nucleic acid and protein. Of these, the former is the more troublesome. If protein is still present, it may be removed by a repetition of the appropriate deproteinization procedure. With many animal tissues the pentose nucleic acid can be completely removed, as nucleoprotein, during the preliminary washing with physiological saline. In other cases, such as the extraction of nucleic acid from rye and wheat[80] and in particular the isolation from bacteria[70], this method is ineffective and the product isolated is a mixture of deoxypentose and pentose nucleic acids. An alternative method for the separation of the acids is therefore required. No really satisfactory procedure has yet been devised, as is illustrated by the wide variety of methods tried. These include: fractionation of the barium salts using glacial acetic acid[8]; fractionation of the calcium salts[71, 81]; electrophoresis[48, 67, 71]; removal of the ribonucleic acid using ribonuclease[71, 81] or by dialysing against alkali[71, 82]; absorption of pentose nucleic acids, if of relatively low molecular weight, on charcoal[83, 84]; and fractional precipitation using cetyltrimethylammonium bromide[84]. Few, if any, of these methods are not open to criticism: the fractionation procedures do not lead to a good separation; electrophoretic separation is laborious and incomplete; action of ribonuclease yields some enzyme-resistant fractions as well as ribonucleotides and alkali degrades the deoxypentose nucleic acid to some extent as well as hydrolysing the pentose nucleic acid to nucleotides. The last two procedures appear to be the most satisfactory.

THE ISOLATION OF PENTOSE NUCLEOPROTEIN

Pentose nucleic acids are more labile to the action of alkali and acid than are deoxypentose nucleic acids; it is therefore equally necessary that the methods for isolation of pentose nucleic acids and nucleoproteins should use only mild reagents. The older methods[85], which employed acid or alkali, yielded degraded products and will therefore not be discussed.

The distribution of pentose nucleic acid in the cell is rather more widespread than that of deoxypentose nucleic acid. While the latter acid is found almost exclusively in the nucleus, the former occurs in both nucleus and cytoplasm, the nuclear pentose nucleic acid being about one-tenth of the total[86]. Most of the pentose nucleic acid of the cytoplasm is present in particulate fractions in the form of nucleic acid–lipoprotein complexes although soluble pentose nucleoproteins are also present. The particulate fractions may frequently be isolated by means of the fractional centrifugation of homogenized tissue which yields the nucleic acid–lipoprotein complex; alternatively the tissue may be extracted with physiological saline which yields a mixture containing the soluble nucleoproteins as well as the particulate material.

Important sources of pentose nucleic acid are the plant and some animal viruses. The viruses, which are largely nucleoprotein, may be isolated either by fractional centrifugation or by salt fractionation.

It would be impracticable to describe all the methods which have been used for the isolation of pentose nucleoprotein and no one method is of wide application, as is true for the deoxypentose nucleoprotein. A selection of typical preparations is therefore given.

Extraction with 0·14M *sodium chloride*[87, 88]

Three hundred and fifty grammes of calf thymus are chopped and suspended in 1·6 l. of 0·14M sodium chloride solution and homogenized in a high speed mincer. The mixture is then stirred for several hours and centrifuged. To the slightly opalescent supernatant 10 per cent acetic acid is added to bring the pH to 4·2, when a copious precipitate appears. The sediment is isolated by centrifuging and washed with 0·5M sodium chloride solution and then with water until free from chloride. The sediment is then suspended in water and dissolved by the careful addition of 0·5M sodium bicarbonate. The volume of the mixture is then adjusted to 500 ml. The solution is clarified by centrifugation at 10,000 rev/min and then precipitated at pH 4·2. This procedure is repeated twice. The final solution is dialysed against water and the nucleoprotein precipitated at pH 4·2, washed with 70 per cent, then 100 per cent ethyl alcohol and dried in a vacuum or air.

The washing procedure with 0·5M sodium chloride is to remove deoxypentose nucleoprotein which contaminates the first precipitate. Kerr and Seraidarian[88] show that this laborious procedure may be omitted if the glands are not homogenized in a high speed mincer but merely ground in a meat chopper, in which case no deoxypentose nucleoprotein is found in the sodium chloride extracts. The reprecipitation procedure from sodium bicarbonate solution can also be avoided in this way, which is advantageous as this procedure involves some loss of nucleic acid from the nucleoprotein[88].

The original isolation of tobacco mosaic virus by Stanley[89] was achieved by salt fractionation and the method has since been used with success for the preparation of several virus nucleoproteins. The method has not been used extensively for the isolation of nucleoproteins although Hamoir has satisfactorily prepared crystalline nucleotropomyosin from carp muscle[90]. An excellent example of this procedure is the isolation of turnip yellow mosaic virus by Markham and Smith[91]. This virus is of particular interest as the pentose nucleic acid component is readily obtained on denaturation of the protein by ethyl alcohol and composes a relatively large proportion of the virus (22 per cent).

The virus has been prepared from a number of cruciferous plants, namely radish, *Brassica carinata*, turnip, Chinese cabbage and from a specimen of broccoli found infected naturally. For general preparation either turnip or Chinese cabbage is suitable and, in general, old or ' hard ' plants give better yields than rapidly growing ' soft ' plants. Yields of up to 1 g of virus per litre of sap have been obtained from stunted turnip plants grown in the open but the usual yields for glasshouse plants vary from 200 to 500 mg/l.

Salt fractionation[91]

Plants infected with virus are minced, either fresh or after freezing overnight, and the sap expressed. If field turnips are used it is necessary to use a hydraulic press to remove the last half of the sap. The pH of the sap is usually about 5·7. The sap is clarified by adding slowly with stirring 300 ml. of 90 per cent ethyl alcohol to each litre. This quantity of alcohol is fairly critical and should be measured carefully. The copious precipitate which forms is centrifuged off at once at low speed (15 minutes at 3500 rev/min). To the supernatant liquid a volume of saturated ammonium sulphate equal to half the volume of clarified sap is added and the solution is allowed to stand, preferably overnight. After a few hours large numbers of small octahedral crystals are to be found in the liquid and these increase in size with time. Many small, highly refractile birefringent crystals are also to be seen ; these are inorganic and are removed later.

The crystalline precipitate is collected by centrifugation (30 min at 3500 rev/min), but the supernatant frequently deposits a second crop of crystals on standing for a day or two, and so may be kept. In addition it is very difficult to remove all the small crystals by centrifuging, as they separate rather slowly because they are not very dense. The precipitate which contains the virus and much insoluble material is resuspended in a small volume of water (one-tenth to one-quarter of the original sap volume) or buffer solution and is centrifuged again to remove insoluble material. Precipitation of the virus followed by redissolving the crystals and centrifuging to remove debris is repeated several times. In favourable circumstances the virus preparation may be fairly clean at this stage.

Several methods may be used for further purification. The virus is now digested by trypsin and treatment with commercial pancreatic extract (10 mg/ml.) and incubation for a few hours with a few drops of chloroform as preservative, will remove some of the contaminants. The enzyme is removed by several recrystallizations of the virus.

A brown pigment is frequently present and this may be removed by crystallization of the virus by dilute ethyl alcohol solutions. A solution containing about 5 mg/ml. of virus and a trace of sodium chloride is cooled to 0°C and 0·25 to 0·30 volume of 100 per cent ethyl alcohol added gradually with stirring. On adding drop by drop a solution containing 20 ml. of 100 per cent ethyl alcohol, 10 ml. of glacial acetic acid and water to make 100 ml., the virus solution becomes turbid, the turbidity disappearing on allowing the solution to warm. If the solution is left at 0°C for a few hours the virus crystallizes out in the form of small birefringent needles, which are centrifuged off in the cold (they dissolve at room temperature when freshly formed). Alternatively they may be left at 1°C for a few days, when the mass of crystals will be found to adhere to the bottom of the container and the mother liquor is decanted without difficulty. The crystals are then dissolved in 0·1M disodium phosphate solution or a neutral buffer, as they form cloudy suspensions in plain water, and the pigmented material, which is now insoluble, removed by centrifugation at 5000 rev/min.

Isolation by fractional centrifugation has been used both for the isolation of cytoplasmic nucleoproteins[92] from the mechanically disrupted cells and also for the isolation of plant viruses from leaves[93-95].

Fractional centrifugation

This method will be illustrated by a description of the preparation of cucumber virus 4[93]. The inoculated plants are harvested and frozen at − 12°C. After a few days the plants are ground. Three per cent by weight of dipotassium phosphate in a 50 per cent solution is mixed with the pulp and after 2 hours the juice expressed from the cold, but completely thawed pulp. The juice is passed through a Celite filter to remove coarse particles of green pigment and extraneous matter, and centrifuged at 20,000–30,000 rev/min for 30 minutes when the virus is sedimented as pellets. The supernatant liquid, which is practically free of virus, is discarded and the pellets dissolved in small amounts of distilled water. The combined solutions of virus pellets are spun at about 3000 rev/min in an angle centrifuge for 30 minutes to remove green pigment and insoluble colloidal matter. The supernatant liquid, which contains the virus, is then returned to the high speed centrifuge and the process repeated about three times, a longer period being allowed for the high speed centrifugation as the virus becomes more concentrated. In many cases it is found possible to effect a better separation of green pigment from the virus by dissolving the pellets obtained by the first two high speed centrifugations in 0·1M phosphate buffer at pH 7 and then using distilled water as solvent for the pellets of the last two centrifugations. All of the preparations used for elementary analysis are further purified by dialysis against flowing distilled water for 48 hours.

The yield of pellets of purified virus is from 0·1–0·4 g/l. of expressed juice.

THE ISOLATION OF THE SODIUM SALT OF PENTOSE NUCLEIC ACID

The requirements for a satisfactory preparation of pentose nucleic acid are very similar to those already described for deoxypentose nucleic acid. The earlier preparations were usually made from yeast which is particularly rich in this nucleic acid. However, in order to extract the pentose nucleic acid the wall of the yeast cell has to be broken. The procedure generally adopted in the past involved the use of alkaline solutions and it is not surprising, therefore, that the preparations were generally of low molecular weight. More recently several mild procedures have been developed which yield a more highly polymerized nucleic acid. However, depolymerization of the pentose nucleic acid can occur during extraction not only by chemical but also by enzymic action, since the depolymerizing enzyme, ribonuclease, is generally found along with the nucleic acid. In replacing the more drastic chemical methods by milder procedures it must be realized that the chances of enzymic degradation are increased. Bacher and Allen[96] have clearly demonstrated that enzymic degradation can occur during the isolation of pentose nucleic acids.

The original method of Clarke and Schryver[29] in which defatted, ground yeast was extracted with 10 per cent sodium chloride at a temperature of 60–80°C was used by Davidson and Waymouth[97] for the extraction of the pentose nucleic acid from sheep liver and later modified by Chargaff and co-workers[98] for the isolation of pentose nucleic acids from the liver of various animals and also from bakers' yeast.

A general method for preparing pentose nucleic acid from animal tissues was described by Volkin and Carter[99], which makes use of the solubility of protein in 2M guanidine chloride solution from which pentose nucleic acid precipitates in the cold. This method has been modified by Grinnan and Mosher[100]. The use of anionic detergents for the precipitation of the protein from a solution of the nucleoprotein, so successfully employed in the preparation of the deoxypentose nucleic acids, has been developed as a general method of preparing pentose nucleic acids from animal tissue by Kay and Dounce[101]. The isolation of pentose nucleic acid from yeast has proved difficult in view of the need to break the cell wall in order to extract the nucleic acid. Crestfield, Smith and Allen[102] and later

Davis and Allen[103] have devised a method which combines hot extraction with buffer solutions, with the simultaneous treatment of the cells with sodium dodecyl sulphate. This procedure yields a white tough fibrous material of apparently high molecular weight and which represents 60 to 70 per cent of the pentose nucleic acid of yeast.

A new method in which extraction with a phenol solution is employed has recently been described for isolating pentose nucleic acids from tissues and from tobacco mosaic virus[104–108]. This method appears to have the advantages of inhibiting ribonuclease action thus producing a highly polymerized product, and of completely removing the protein and deoxypentose nucleic acid during the course of the extraction. The only difficulty appears to be the complete removal of polysaccharide.

The preparation of pentose nucleic acids from plant viruses has been carried out by a variety of different methods; the separation of the protein appears to be easier in some viruses than others. Cohen and Stanley[109], in their important study of the nucleic acid of tobacco mosaic virus, denatured the protein thus rendering it insoluble by heating. Dorner and Knight[110] have made a systematic study of the preparation of pentose nucleic acids from a variety of plant viruses. The methods tried on tomato bushy stunt virus included treatment with acids, bases, alcohol, detergents, proteolytic enzymes, sonic vibration, heat and cold, salt, butanol and chloroform, guanidine hydrochloride and condensed gases at low temperatures. Only two methods gave reasonable yields, freezing and thawing for 60 cycles followed by salting out of the protein, and treatment with sodium dodecyl sulphate (Duponol C) at 100°C followed by salting out of the protein. The freezing and thawing method gave a highly inhomogeneous product and was therefore abandoned, whereas the sodium dodecyl sulphate method gave satisfactory results. A similar method described by Bawden and Pirie[111], in which lower temperatures were employed, was not found to be satisfactory by Dorner and Knight. The turnip yellow mosaic virus is denatured at room temperature by adding ethyl alcohol to produce a concentration of 33 per cent[91]; this affords a method of preparing the nucleic acid in neutral cold solutions. In aqueous, faintly alkaline solutions this pentose nucleic acid forms clear highly viscous solutions, suggesting that the nucleic acid is highly polymerized.

Details of five procedures are given below. The method adopted must depend very much on the type of tissue which is being extracted.

Preparation of sodium pentose nucleate from mammalian tissue using guanidine hydrochloride[99]

The method of isolation of pentose nucleic acid from tissue homogenates consists of (*1*) the removal of deoxypentose nucleic acid as a nucleic acid–protein complex, (*2*) the precipitation of the pentose nucleic acid from a cold 2M guanidine hydrochloride solution in which the large bulk of protein remains soluble, and (*3*) further purification of the pentose nucleic acid by the chloroform-gel method.

Fresh frozen tissue is cut into small pieces and homogenized in a high speed mincer for 6 to 8 minutes with three volumes per gramme of tissue of a 0·15M sodium chloride–0·02M phosphate buffer at pH 6·8. A few drops of octyl alcohol are added to reduce foaming. The homogenate is centrifuged at 3000*g* for 30 minutes. Essentially all the deoxypentose nucleic acid is removed at this stage as insoluble nucleic acid–protein complex.

To the supernatant solution, solid guanidine hydrochloride is added, with rapid stirring to give a 2M solution. The solution is kept at 38°C for 30 minutes and then chilled at 0°C for 1 hour. Under these conditions most of the protein of the tissue extract remains soluble, while a gelatinous precipitate forms which contains pentose nucleic acid and a small amount of protein. The precipitate is washed twice with a cold solution of 2M guanidine hydrochloride in the sodium chloride–phosphate buffer of pH 6·8. This removes any deoxypentose nucleoprotein which remains in solution in the concentrated guanidine hydrochloride solution. To remove contaminating protein, the precipitate is suspended in 2M guanidine hydrochloride (one volume per gramme of original tissue) and extracted with chloroform–octyl alcohol (ratio 5 : 1) by the Sevag, Lackman and Smolens procedure[15]. The suspension of nucleic acid in guanidine hydrochloride is added to an equal volume of the chloroform–octyl alcohol mixture, warmed to 40°C and shaken mechanically for 30 minutes. The mixture is centrifuged and the upper aqueous layer containing the nucleic acid removed. This extraction is repeated twice with fresh chloroform–octyl alcohol mixture.

It is necessary to carry out these extractions at 40°C, as extractions in the cold, or in sodium chloride or water solutions result in incomplete separation of the nucleic acid from the protein. Nucleic acid is precipitated in the cold from the guanidine hydrochloride solution by adjusting the acidity to a pH value between 4·2 and 4·5 with acetic acid and adding two volumes of cold ethyl alcohol. The white flocculent pentose nucleic acid precipitate is centrifuged and washed twice with 70 per cent ethyl alcohol. The precipitate is then dissolved in water, carefully adjusted to pH 6·8 with dilute sodium hydroxide, and any insoluble denatured protein removed by centrifugation. The nucleic acid is purified by adding 1M sodium chloride solution to bring the final concentration to 0·05M sodium chloride and precipitating the sodium pentose nucleate with two volumes of ethyl alcohol. The product is washed twice with 70 per cent ethyl alcohol.

An alternative procedure which prevents the possibility of any nuclease action, involves the immediate homogenization of the fresh tissue with three volumes per gramme of tissue of cold 2·5M guanidine hydrochloride solution. The rest of the procedure follows the above method, except that the pentose nucleic acid–protein complex is washed at least three times with cold 2M guanidine hydrochloride solution to ensure the removal of contaminating deoxypentose nucleic acid.

Kay and Dounce[101] criticized the Volkin and Carter procedure[99] on the grounds that even after the separation of protein from the nucleic acid by means of guanidine hydrochloride, further purification using the chloroform-gel method was necessary. They therefore devised the following method which they claim gives a highly polymerized pentose nucleic acid with no detectable protein.

Preparation of sodium pentose nucleate from mammalian tissue using sodium dodecyl sulphate[101]

Fifty grammes of fresh frozen tissue are chopped into small pieces and homogenized in a high speed mincer (Waring blendor) with 200 ml. of an ice-cold solution containing 0·9 per cent sodium chloride and 0·01M sodium citrate for 5 minutes at between 0 and 3°C. The homogenate is centrifuged at 0–3°C at 2500 rev/min for 30 minutes to remove insoluble deoxypentose nucleoproteins. The supernatant is decanted and is centrifuged again at 2500 rev/min in the cold to further remove any residual deoxypentose nucleoproteins. The supernatant fluid is decanted and to it is added, with stirring in the cold, enough 1N hydrochloric acid to lower the pH to 4·5. The heavy precipitate is centrifuged at 2500 rev/min for 15 minutes in the cold. The red supernatant is discarded. The sediment is mixed with sufficient 0·9 per cent sodium chloride solution to make a total volume of 100 ml. Nine ml. of a 5 per cent solution of purified sodium dodecyl sulphate in 45 per cent ethyl alcohol are added with stirring. A 10 per cent solution of sodium hydroxide is carefully and slowly added to the mixture to raise the pH to 7, and the stirring is continued for 3 hours. Sufficient solid sodium chloride is then added to make the concentration 1M with respect to sodium chloride. The slightly viscous solution is centrifuged for 30 minutes at 14,000 rev/min (Servall centrifuge); the supernatant is decanted and the nucleic acid is precipitated by adding two volumes of 95 per cent ethyl alcohol. The white flocculent precipitate which forms is centrifuged, washed with ethyl alcohol and acetone and dried in air.

The dry material is dissolved in 100 ml. of water and 9 ml. of the sodium dodecyl sulphate solution added with stirring. The solution is stirred for 2 hours at room temperature. Sufficient solid sodium chloride is added to make the concentration 1M with respect to sodium chloride. The solution is centrifuged at 14,000 rev/min for 30 minutes. The supernatant is then decanted and the nucleic acid precipitated by adding two volumes of ethyl alcohol. The white flocculent precipitate is centrifuged and washed with ethyl alcohol, acetone and dried in air.

The white powder from the above procedure is dissolved in 30 ml. of water and the solution is cooled to about 0°C. Sodium chloride (solid) is added to bring the solution to 0·9 per cent and the pH is lowered to 4·5 by the careful addition of 0·1N hydrochloric acid. The solution is centrifuged, in the cold, at 14,000 rev/min for 1 hour. The supernatant is decanted into a beaker containing enough solid sodium chloride to make the final concentration of sodium chloride 1M. When the salt has completely dissolved, the pH of the solution is raised to 7·0 by the careful addition of 0·1N sodium hydroxide to the still cold solution. The nucleic acid, as its sodium salt, is then precipitated by the addition of two volumes of 95 per cent ethyl alcohol. The white flocculent precipitate is collected by centrifugation, washed with ethyl alcohol and acetone and finally dried in air.

The final product is a white powder which dissolves readily in water to give a clear solution. The yield is about 200 mg (dry weight) from 50 g of rat liver (wet weight). This yield represents about 50 per cent of the tissue pentose nucleic acid.

Preparation of sodium pentose nucleate from mammalian tissue using phenol[106]

Rat livers are homogenized in a high speed mincer in water for 60 seconds. The homogenate is filtered through a Buchner funnel to remove fibrous tissue and an equal volume of phenol solution (90 per cent w/v) added rapidly with stirring. Stirring is continued for 1 hour, and then the mixture is centrifuged at 1750 rev/min for 1 hour at 0°C. The system in the centrifuge tube consists of two layers, the top aqueous layer contains all the pentose nucleic acid together with polysaccharide, while the bottom phenol layer contains the deoxypentose nucleic acid and protein. Insoluble protein exists at the phenol–water interface. The cloudy aqueous layer is removed by suction and the phenol layer washed three times with water. The combined aqueous extracts are made up to 2 per cent with respect to potassium acetate and the pentose nucleic acid precipitated by the addition of two volumes of ethyl alcohol. The precipitate is centrifuged (1750 rev/min for 20 minutes), washed once with ethyl alcohol–water (3 : 1) and dissolved in 25–100 ml. of water. Ethyl alcohol is removed either by a rotary evaporator or by washing with ether and removing the ether with a stream of nitrogen.

The clear aqueous solution is mixed with an equal volume of 2·5M potassium hydrogen phosphate (K_2HPO_4), 0·05 volume of

33·3 per cent phosphoric acid and one volume of 2-methoxyethanol (redistilled). The lower layer together with any insoluble material is separated either by standing or centrifuging and washed once with the top layer from a mixture of 2-methoxyethanol–water–2·5M potassium hydrogen phosphate–33·3 per cent phosphoric acid (1 : 1 : 1 : 0·05, by volume). The combined top layers contain all the pentose nucleic acid and any insoluble matter can be removed by centrifugation in Polythene tubes at 10,000*g* for 1 hour. The clear supernatants are combined, a few drops of toluene added and the whole is dialysed against water (2 l.) which is changed at least four times. The contents of the dialysis bag are centrifuged if necessary, made up to 2 per cent with respect to potassium acetate and the sodium pentose nucleate precipitated by the addition of two volumes of ethyl alcohol. The precipitate is collected by centrifuging, washed two or three times with ethyl alcohol–water (3 : 1) and dried in a vacuum over phosphorus pentoxide. Yield of material containing about 12 per cent of water is 450–650 mg/100 g of rat liver.

According to Crestfield, Smith and Allen[102], the method described below is designed specifically to eliminate enzymic degradation during isolation. They consider that the product isolated is significantly different and presumably superior to, other specimens.

Preparation of ribonucleic acid from yeast[102]

A 4-l. beaker is fitted with a top plate that contains a narrow slot through which a motor-driven stirring rod and thermometer can be introduced. Five hundred ml. of a solution which contains 2 per cent sodium dodecyl sulphate, 4·5 per cent ethyl alcohol, 0·0125M sodium dihydrogen phosphate and 0·0125M disodium hydrogen phosphate are placed in the beaker (pH 6·5 to 6·7). The solution is brought to boiling with stirring. The top plate is removed and with constant stirring, 150 g of yeast cut into very fine pieces are added to the boiling solution. The top plate is immediately replaced to prevent loss of water by evaporation. The temperature after the addition of the yeast ranges from 83 to 87°C. Heating is continued with stirring for 1 minute, after which the beaker and contents are transferred to a boiling water bath for 2 minutes in order to avoid excessive foaming. At the time of transfer to the water bath, the temperature of the solution ranges from 92 to 94°C. After 2 minutes on the water bath, the contents of the beaker are poured into a 2-l. beaker which is immersed in a solid carbon dioxide–cellosolve freezing mixture. The contents of the beaker are stirred manually for 4 to 8 minutes until the temperature drops to approximately 4°C. The mixture is centrifuged at 2000 rev/min for 30 minutes at 0°C.

The supernatant is poured with stirring into two volumes of cold ethyl alcohol. The precipitate is collected by centrifugation at 2000 rev/min in the cold for approximately 15 minutes and washed with two 150-ml. portions of cold 67 per cent ethyl alcohol. In washing it is advisable to homogenize the precipitate with a glass rod and add the wash liquid slowly. Five to ten drops of a 2N solution of sodium chloride are added to ensure flocculation and recovery; 15 to 20 minutes centrifugation at 2000 rev/min are sufficient for each step in the washing. The crude nucleic acids are suspended in 80 per cent ethyl alcohol and permitted to stand overnight in the cold to aid the removal of protein. This crude product contains over 80 per cent of the original ribonucleic acids present in the yeast.

The crude nucleic acids are collected by centrifugation and dissolved in 130–180 ml. of water. The solution is turbid, with a pH of approximately 8. The solution is immediately neutralized to pH 7·0 by the careful addition of 1N acetic acid. Turbidity is removed by centrifugation for 30 minutes at 20,000 rev/min at 0°C (Spinco model L preparative centrifuge). A certain amount of floating material is not removable at this point. Solid sodium chloride is added to the supernatant to bring the final concentration to 1M. Upon standing at 0°C, the turbidity increases slightly and the viscosity increases markedly. In about 30 minutes a gel separates. The supernatant fluid, which is yellow and contains a small amount of floating material, is separated from the gel by centrifugation at 2000 rev/min for 1 hour. The supernatant liquid is discarded. The gel is washed successively with three 150-ml. portions of 67 per cent ethyl alcohol, to each of which has been added 1 ml. of a 2M solution of sodium chloride. The washed precipitate is dissolved by the slow addition of water with continuous stirring. The pH of the solution is 7·0. It is dialysed for 36 hours at 4°C against frequent changes of distilled water. Turbidity is removed, first by filtration through Celite and finally through an asbestos pad (D-7 Steriflo, F. R. Hormann & Co., Newark, N.J., U.S.A.). The clear colourless solution is lyophilized to yield a white solid. The yield is 1·4 g, which is equivalent to 60 to 70 per cent of the original ribonucleic acid present in the yeast.

The following preparation described by Dorner and Knight[110], was used by them to obtain the pentose nucleic acid from tomato bushy stunt virus (BS), southern bean mosaic virus (SBM) and potato virus X (PX).

The preparation of pentose nucleic acids from plant virus[110]

Sodium dodecyl sulphate (Duponol C) solution is added to virus solution so that each ml. of reaction mixture contains respectively, 10 mg of sodium dodecyl sulphate and 15 mg of BS, 16 mg of sodium dodecyl sulphate and 7 mg of SBM, and 2·5 mg of sodium dodecyl sulphate and 1·5 mg of PX. Each mixture is heated in centrifuge tubes in batches of about 40 ml. in a boiling water bath for 4 minutes and is then chilled in an ice bath, after which it is dialysed against several changes of distilled water for 4·5 hours. The dialysed preparation is then made 1M with respect to sodium chloride by adding an appropriate amount of 5M sodium chloride solution and heated at 100°C for 3 minutes. After chilling in an ice bath, the protein precipitate is centrifuged off and washed several times with hot 1M sodium chloride and the combined supernatant fluids and washings dialysed against several changes of distilled water for 15 to 24 hours. With SBM and PX it is found necessary to repeat the precipitation of protein with 1M salt after concentration of the solution to about 30 ml. and to dialyse for another 15 to 24 hours. In all three cases the dialysed preparation is concentrated 20- to 70-fold by directing a stream of air at the solution held in a cellophane bag; the concentrate is then centrifuged at 40,000 rev/min (Spinco model L preparative centrifuge) for 1 hour. A small pellet of denatured protein is generally obtained at this stage and from it the clear supernatant is poured off. The nucleic acid is then obtained by lyophilizing the solution.

The only two methods of isolating pentose nucleic acids that are really capable of comparison are those of Volkin and Carter[99] using guanidine hydrochloride and of Kay and Dounce[101] employing sodium dodecyl sulphate. No systematic study of the physico-chemical properties of the products isolated by these methods has been made, but in view of the known fractionation incurred by the use of the chloroform-gel deproteinization method for deoxypentose nucleic acids[78], which would also certainly apply to pentose nucleic acids, the latter method is to be preferred.

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3

THE HETEROGENEITY AND FRACTIONATION OF NUCLEIC ACIDS

INTRODUCTION

There are a great many heritable factors in the total genetic complement of any cell. It is therefore necessary, if the identity between the active part of the gene and deoxypentose nucleic acid is correct, to postulate that the total deoxypentose nucleic acid of the cell consists either of a great many different nucleic acid molecules each concerned with a single genetic function and having a characteristic chemical structure, or of relatively few macromolecules which possess specific structures along the chain, each such structure forming a region having a particular genetic function[1]. It is not known which of these two alternatives obtains, or whether both methods are used to form the genetic code, but irrespective of the method used there will almost certainly exist both chemical and physical heterogeneity in a sample of the total deoxypentose nucleic acid isolated from a cell.

Heterogeneity can also be expected in pentose nucleic acids. Considerable evidence has been accumulated which points to an intimate association between pentose nucleic acid and protein synthesis. If such a relationship exists, the very large number of different proteins which are known to be synthesized by a single cell must indicate an inherent heterogeneity in the pentose nucleic acids of the cell. Therefore, as with deoxypentose nucleic acid, the total pentose nucleic acid isolated from a cell will in all probability exhibit both physical and chemical heterogeneity. The division of samples of nucleic acids into fractions by either chemical or physical means, must now be regarded as a necessary preliminary step in any detailed study of nucleic acid structure and clearly must precede any attempt to determine the sequence of nucleotides in the polynucleotide chain.

The great majority of studies so far carried out, have been concerned with the heterogeneity and fractionation of deoxypentose nucleic acids rather than pentose nucleic acids. This is a reflection of the present view that the genetical functions in a cell are controlled by the deoxypentose nucleic acid of the nucleus whereas the function of the pentose nucleic acid in the cytoplasm is less clear. Furthermore, satisfactory and proven methods of preparing deoxypentose nucleic acid have been known for somewhat longer than those of pentose nucleic acid.

THE PHYSICAL HETEROGENEITY OF DEOXYPENTOSE NUCLEIC ACID

The heterodisperse nature of the deoxypentose nucleate ion in solution has long been suspected, although direct quantitative evidence has only been obtained recently. The sedimentation of nucleic acids using a Schlieren (Philpott–Svensson) optical system can only be studied at concentrations down to 5×10^{-4} g/ml. In this concentration range a hypersharp Schlieren boundary, frequently observed with solutions of polyelectrolytes, is obtained. Although the cause of the formation of the hypersharp boundary is obscure, there can be no doubt that the shape of the boundary bears no relation to the distribution of sedimentation coefficients of the sample of nucleic acid.

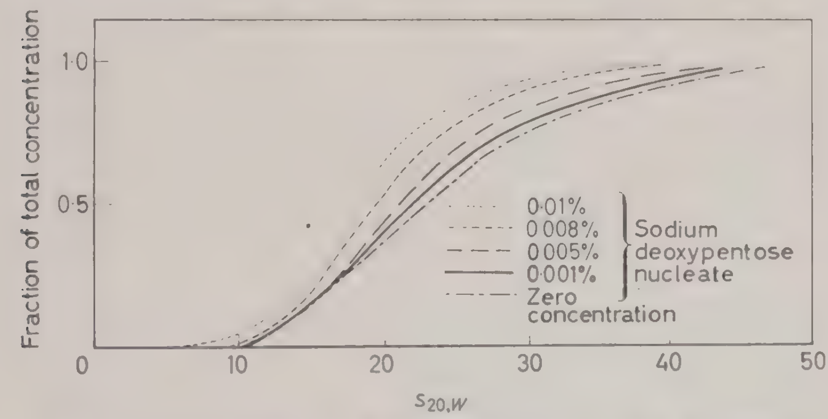

Figure 1. Sedimentation coefficient distribution curves for different concentrations of sodium deoxypentose nucleate in 0·2M sodium chloride (K. V. Shooter and J. A. V. Butler: *Trans. Faraday Soc.* 1956, **52**, p. 739)

By employing an ultra-violet absorption optical system, use may be made of the strong absorption in the ultra-violet shown by solutions of nucleic acids and the sedimentation coefficients at concentrations down to 10^{-5} g/ml. may thus be determined. By means of this method Shooter and Butler[2, 3] have measured the sedimentation coefficient distribution for solutions of sodium deoxypentose nucleate from the variation of the ultra-violet absorption, and therefore of nucleate concentration, across the sedimenting boundary. The variation of the sedimentation coefficient distribution with concentration of nucleate is shown in *Figure 1.* The curves show that the concentration dependence of the sedimentation coefficient is small for material having low values of the sedimentation coefficient but is greater at higher values. The important relationship, however, is that at zero concentration (calculated by the method of Oth and Desreux[4]) which gives the true distribution of sedimentation coefficients.

Comparison of the sedimentation coefficient distribution of the deoxypentose nucleic acid with that of the parent nucleoprotein[5] shows that using the anionic detergent procedure for isolating the nucleic acid (the method of Kay, Simmons and Dounce, see Chapter 2, ref. 60), there is no change in the distribution and no fractionation has taken place during the deproteinization process. However, deproteinization by the chloroform-gel method (the method of Sevag, Lackman and Smolens, see Chapter 2, ref. 15) yields a nucleic acid with a markedly different sedimentation coefficient distribution curve from that of the original nucleoprotein, indicating that fractionation of the deoxypentose nucleic acid had occurred during the deproteinization process (*Figure 2*).

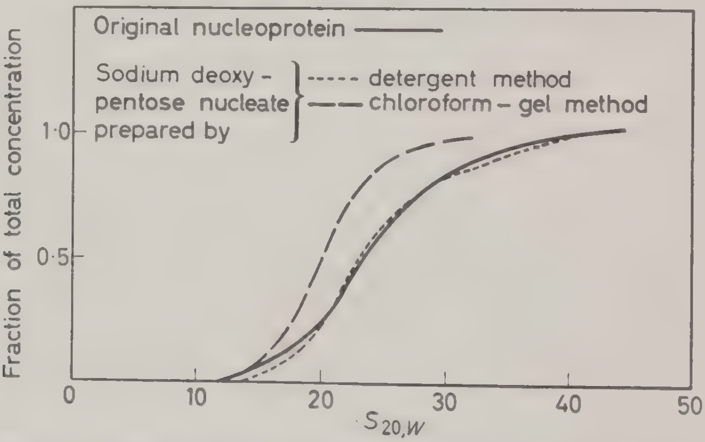

Figure 2. Sedimentation coefficient distribution curves for different preparations of sodium deoxypentose nucleate and for nucleoprotein

(K. V. Shooter and J. A. V. Butler: *J. Polym. Sci.* 1957, **23,** p. 707)

The observation of such marked heterogeneity in solutions of sodium deoxypentose nucleate by sedimentation methods is somewhat surprising in view of the fact that for a system of rigid rods of constant cross-section, the sedimentation coefficient should be almost independent of the length of the rods/[7,8]. The marked heterogeneity observed by Shooter and Butler suggests that either the deoxypentose nucleate ions are to some extent flexible or that their diameter is variable, possibly on account of aggregation. To confirm unambiguously that the observed heterogeneity was not due to aggregation, Shooter and Butler[6] fractionated the nucleate in a swing bucket rotor at 39,000 rev/min in a Spinco model E ultracentrifuge for 4·5 hours. The sedimentation was continued until the solution concentration, originally 7×10^{-5} g/ml. became 1×10^{-5} g/ml. and all material with sedimentation coefficients greater than 20 had been sedimented to the bottom of the tubes. On standing, no aggregation, as determined by sedimentation, was observed in this solution. A comparison of the sedimentation coefficient distribution for this fraction with that of the original deoxypentose nucleate is shown in *Figure 3* and although there is still a range of sedimentation coefficients this is clearly less than in the original material.

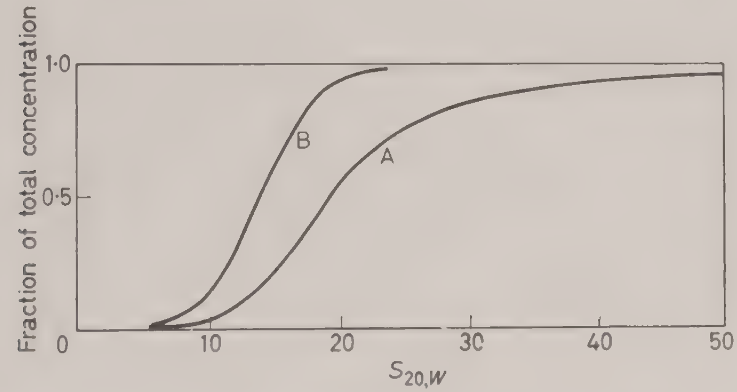

Figure 3. Sedimentation coefficient distribution curves of sodium deoxypentose nucleate showing fractionation achieved after prolonged centrifugation
A—original solution ; B—fraction obtained after centrifuging
(K. V. Shooter and J. A. V. Butler: *J. Polym. Sci.* 1957, **23,** p. 706)

THE CHEMICAL HETEROGENEITY AND FRACTIONATION OF DEOXYPENTOSE NUCLEIC ACID

In the attempt to separate deoxypentose nucleic acid into fractions of discrete genetical activity a wide variety of methods has been employed. Probably the earliest suggestion that fractionation of deoxypentose nucleic acid is possible was that of Mirsky and Ris[9] who observed that while the greater part of the deoxypentose nucleic acid of the isolated chromosomes is soluble in 1M sodium chloride solution, a little nucleic acid was still detectable in the residual chromosomes which were insoluble in this solution. This residual nucleic acid was originally thought to have been a contaminant, but it may well be possible that a fractionation based on the solubility in 1M sodium chloride had been carried out. The same possibility applies to the brief report[10] of the solubility of only part of the deoxypentose nucleic acid of liver nuclei in strong sucrose solution.

The first real attempt at fractionation of deoxypentose nucleic acid was made by Bendich[11] and Bendich, Russell and Brown[12] by utilizing the different solubility of two fractions in 0·87 per cent sodium chloride. Significant differences in the ratio of the soluble to insoluble fraction were observed in nucleic acids isolated from different tissues; the two fractions from any one tissue differed in the extent to which previously administered ^{14}C had been incorporated into the various purines and pyrimidines.

A more extensive procedure, resulting in the isolation of seven fractions, was introduced by Chargaff, Crampton and Lipshitz[13], who, after extracting the nucleoprotein from calf thymus, isolated it as a gel by treating the aqueous solution with a chloroform–*n*-octanol mixture in a high speed mixer. The gel was then extracted, stepwise, with sodium chloride solutions of increasing concentration, the nucleoprotein precipitated with ethyl alcohol, and the deoxyribose nucleic acid extracted by the chloroform-gel method. The fractions were all studied analytically and the most significant observation was that the extraction with sodium chloride solutions of increasing strength yields a series of nucleic acid fractions with diminishing concentrations of guanine and cytosine and rising concentrations of adenine and thymine. Each pair of constituents was, however, present throughout in equimolecular proportions. This method of fractionation is probably based on differences in the stability of the bonds between the nucleic acid and protein and the same authors[14], in a more extensive study, have investigated the part played by artificial protein-nucleates in the fractionation procedure and also extended the investigation to the deoxypentose nucleic acids of other tissues.

Lucy and Butler[15] have repeated the work of Chargaff, Crampton and Lipshitz and while they confirm in general the fractionation procedure, their results show that the essential variable in the extraction of the gel with sodium chloride solutions is not the concentration of sodium chloride, since a similar fractionation was achieved by repeatedly extracting the gel with solutions of the same concentration.

Fractionation of the deoxypentose nucleic acid from bacterial cells has also been attempted[16] using differential solubility in alkaline solutions. Two fractions were obtained, one readily soluble in 1M sodium hydroxide at 37°C while the other remained firmly bound to polysaccharide material under these conditions. Analytical examination of these two fractions showed no difference in the purine and pyrimidine composition.

A significant advance in fractionation procedures came with the attempt to separate the nucleic acid fractions by chromatography. In view of the affinity of nucleic acids for basic proteins and the possibility that differences in chemical structure between nucleic acid molecules may affect the strength of the bond with protein, Brown and Watson[17] used chromatographic columns in which histone, prepared from calf thymus, was immobilized on the surface of kieselguhr. The histone-coated kieselguhr was dispersed in 0·4M sodium chloride and solutions of deoxyribose nucleic acid (calf

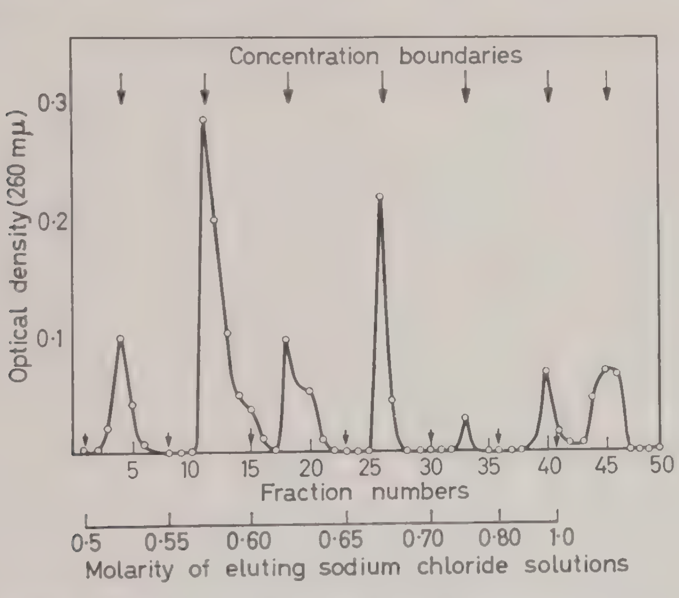

Figure 4. Discontinuous elution of deoxyribose nucleic acid of calf thymus with solutions of sodium chloride from a column of histone-coated kieselguhr

(G. L. Brown and M. Watson: *Nature, Lond.* 1953, **172,** p. 340) thymus) at suitable concentrations added to the top of the chromatographic column. These were washed into the column under pressure, where the nucleic acid was adsorbed. The nucleic acid was then eluted by stronger sodium chloride solutions, either by stepwise increases in sodium chloride concentration or with continuously increasing concentrations. A typical stepwise elution diagram is shown in *Figure 4.* This shows clearly that with each increase of sodium chloride concentration, a definite fraction of deoxyribose nucleic acid is eluted sharply at the concentration boundary between one eluting solution and that preceding it, thus demonstrating that the preparation is heterogeneous with respect to the strength of the salt linkages binding the nucleic acid to the basic protein. Analysis of the fractions showed that the ratios of adenine to guanine and of thymine to cytosine in the nucleic acid fractions increased with increase in the sodium chloride concentration of the eluting solution, thus confirming the previous results of Chargaff, Crampton and Lipshitz[13]. Brown and Watson also were able to demonstrate that the elution diagram varied markedly with nucleic acids from different sources. In place of kieselguhr as the supporting agent for the histone, Brown and Martin[18] employed columns of phosphorylated cellulose prepared by the method of Hoffpauir and Guthrie[19]. The histone was fractionated on the column and a selected fraction attached to the cellulose, by denaturing the protein by heat followed by diazotization[20]. Employing this column, bacteriophage deoxypentose nucleic acid was separated into two distinct fractions[18, 21]. This fractionation was independent of the method of preparing the nucleic acid from bacteriophage and the two fractions had quite distinct adenine to guanine ratios. A similar chromatographic procedure was used by Lerman[22] to fractionate the transforming principle of pneumococcus.

Calcium phosphate gel, which has been satisfactorily used for the chromatography of proteins[23] and of enzymes, has been employed by Main and Cole[24], and by Semenza[25] for the fractionation of deoxypentose nucleic acid. The preparation of the columns has been described by Tiselius and co-workers[23, 26]. Solutions of nucleic acid in phosphate buffer were eluted stepwise[25] or by linear gradient elution. Reproducible fractionation into three elution peaks was observed by Semenza, but Main and Cole obtained no multiple elution peaks and observed no significant fractionation.

The most satisfactory fractionation technique so far developed is that of Bendich and his collaborators[1, 27–31]. Since deoxypentose nucleic acid carries a net negative charge due to the ionized phosphate groups (the isoelectric point is below pH 1), they have treated the nucleic acid as a mixture of polyanions and accordingly used the method of anion-exchange chromatography. The choice of a suitable exchanger proved difficult and several were tried before a satisfactory fractionation could be effected[31]. The strong base resin, Dowex-1, which has proved useful in the fractionation of nucleotides (see Chapter 4) shows a low capacity for deoxypentose nucleic acid and that fraction of the nucleic acid that is absorbed by the resin is bound too lightly for useful chromatography. More promising results were obtained with the weak base resin, Amberlite IR4B. By eluting with sodium chloride solutions of increasing ionic strength, much of the absorbed nucleic acid may be recovered from this exchanger as numerous elution fractions (between 16 and 200 elution peaks were observed in different experiments). However, the Amberlite resin is not very satisfactory in other directions, the results obtained are not reproducible and large amounts of resin impurities are discharged into the eluate.

The most encouraging results have been obtained with certain nitrogen-containing cellulose derivatives, which were developed by Peterson and Sober[32–35] for the fractionation of proteins and peptides. Of the various exchangers tried, that showing most promise for nucleic acid fractionation has been named ECTEOLA-SF by Peterson and Sober. This exchanger, although of little use for proteins and peptides, has been found to be very satisfactory for the fractionation of deoxypentose nucleic acids[1, 27–31, 36, 37]. It is prepared from high purity wood cellulose (Peterson and Sober, and Bendich and collaborators used a preparation known as Solka-Floc*) by treatment with triethanolamine and epichlorohydrin in the following way.

> Sixty grammes of sodium hydroxide in 150 ml. of water are added, with stirring, to 60 g of Solka-Floc and cooled in ice for 30 minutes. A mixture of 35 ml. of triethanolamine and 60 ml. of epichlorohydrin is added in several portions. After thorough mixing, the mixture is heated on an oil bath at 75–80°C for 30 minutes with occasional stirring. Approximately 10 minutes after immersion in the oil bath, an exothermic reaction occurs which is accompanied by a rapid evolution of vapour. At the end of the heating period, the gummy amber mass is cooled in an ice bath and 250 ml. of 2M sodium chloride added in portions, with stirring, to produce a smooth suspension. The product is filtered, washed with alcohol three times and dried in a vacuum.

The exchanger so prepared has a base strength (pK_a') in 0·5M sodium chloride solution of 7·5 and very probably contains cross-linkages involving nitrogen. Provided that the synthetic sequence[34] is carefully followed, the preparation of the exchangers has been found to be reasonably reproducible[31]. Exchangers with different adsorption characteristics may be prepared by using different ratios of triethanolamine and epichlorohydrin in the preparation.

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* Supplied by the Brown Company, 500 Fifth Avenue, New York 36, U.S.A.

A very detailed account of the adsorption properties of ECTEOLA-SF for deoxypentose nucleic acids, oligonucleotides and nucleotides has been given by Bendich, Pahl, Korngold, Rosenkranz and Fresco[31]. The adsorption capacity of this exchanger for deoxypentose nucleic acid is around 8–10 mg/g of exchanger when prepared by the method given above. However, the capacity for mononucleotides is very low, but a mixture of oligonucleotides, prepared by the digestion of calf thymus deoxyribose nucleic acid with crystalline pancreatic deoxyribonuclease was adsorbed sufficiently well to yield a satisfactory fractionation.

Columns of ECTEOLA are best prepared[31] in a tube 2 × 10 cm to which is sealed, at the bottom end, a coarse-porosity sintered Pyrex funnel (0·8 cm internal diameter and 12 cm high) and at the top a standard ground glass socket. The ECTEOLA is poured into the column as a slurry of 0·5 g of ECTEOLA and about 20 ml. of 0·01M phosphate buffer at pH 7·0. The flow rate is controlled by a thumb-screw and rubber tubing attached to the bottom of the column. The exchanger is then washed with 0·5N sodium hydroxide solution at a rate of about 5–10 ml. per hour until the effluent shows a negligible optical density at 260 mμ (less than 0·015). Excessive washing does not appear to harm the exchanger and it is recommended that the washing with alkali is continued for 3 to 4 days. The alkali washing is followed by washing with 0·01M phosphate buffer at pH 7·0 at a similar rate flow for a further 3 to 4 days. The deoxypentose nucleic acid is added in the form of a solution containing 1 g/ml. in either a phosphate buffer at pH 7·0 or in 0·001M sodium chloride. The solution, 1–3 ml., is pipetted slowly into the solution above the freshly washed exchanger. The solution is allowed to percolate through the exchanger under gravity at a rate not exceeding 2–4 ml. per hour. When all but 0·5 ml. of the solution above the column has passed through, the walls are washed down with a few ml. of the buffer or sodium chloride solution and the flow continued until 0·5 ml. remains above the exchanger. Several hundred ml. of the phosphate buffer are now passed through the exchanger, now charged with nucleic acid, at a rate of 3 ml. per hour until the optical density of the effluent is again less than 0·015. The nucleic acid is now strongly bound to the exchanger and is not removed by excessive washing by the phosphate buffer.

Elution of the ECTEOLA–nucleic acid column may be achieved either by using a discontinuous technique or by gradient elution. In the former method the sodium chloride concentration is increased stepwise from 0·05M to 0·1, 0·2, *etc.* until 2M is reached, the flow rate being maintained at 5 to 6 ml. per hour. The ultra-violet absorption in the region of the maximum (from 240–290 mμ) of the eluate is determined and the elution with a given concentration continued until a low constant optical density of the eluate is obtained.

A chromatographic profile (*Figure 5*) showing two large fractions between 0·2 and 0·4M sodium chloride was obtained by Bendich, Fresco, Rosenkranz and Beiser[27], for calf thymus deoxyribose nucleic acid. (The chromatographic profiles are obtained by plotting the optical density at 260 mμ multiplied by the volume of the fraction in ml. against both the tube number and concentration of eluting solution.)

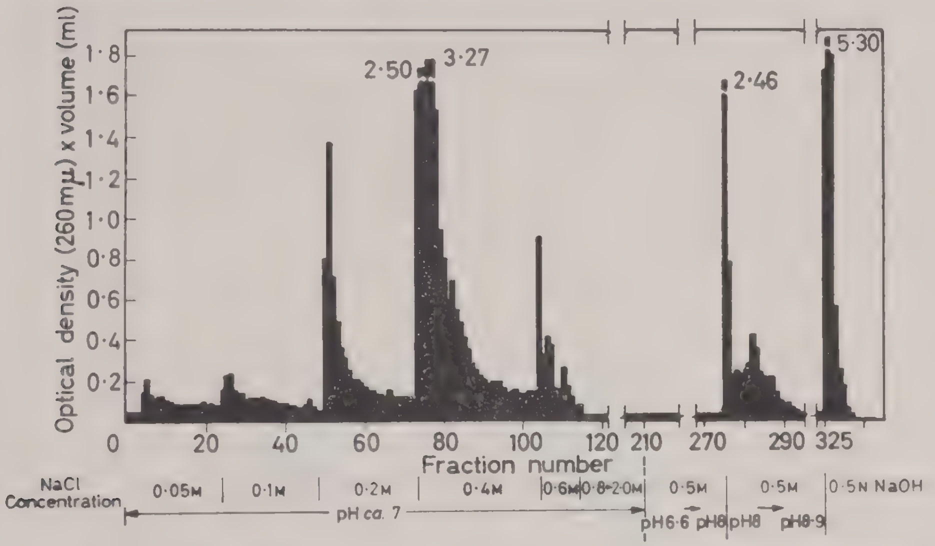

Figure 5. Discontinuous elution of deoxyribose nucleic acid of calf thymus from ECTEOLA-cellulose column (3·6 mg deoxyribose nucleic acid on 0·5 g ECTEOLA-cellulose)

(A. Bendich, J. R. Fresco, H. S. Rosenkranz and S. M. Beiser: *J. Amer. chem. Soc.* 1955, **77**, p. 3672)

Gradient elution is, in general, a more satisfactory technique but Bendich, Fresco, Rosenkranz and Beiser[27] were unable to resolve the two large fractions shown in *Figure 5* when using a single mixing chamber gradient elution system. However, by using two mixing chambers better resolution could be effected. The object of the double mixing chamber system is to achieve the concentration range very gradually. The system, as described by Bendich, Pahl, Korngold, Rosenkranz and Fresco[31], is shown in *Figure 6*.

A stock solution of 0·5M sodium chloride in 0·01M phosphate buffer at pH 7·0 is contained in a dropping funnel and drips into a

150-ml. conical flask containing 100–125 ml. of the phosphate buffer and stirred magnetically. This solution then flows into the second mixing chamber which contains an amount of phosphate buffer equal to that in the first, and is also stirred magnetically. For any small increment of eluent, after the passage of a measured volume of effluent, the sodium chloride concentration may be obtained from the following expression[31]:

$$[\mathrm{NaCl}]_{\mathrm{effluent}} = [\mathrm{NaCl}]_{\mathrm{stock}} - [\mathrm{NaCl}]_{\mathrm{stock}} \frac{V+1}{e^{V}}$$

$$\text{where } V = \frac{\text{cumulative volume passed through}}{\text{volume in mixing chamber}}.$$

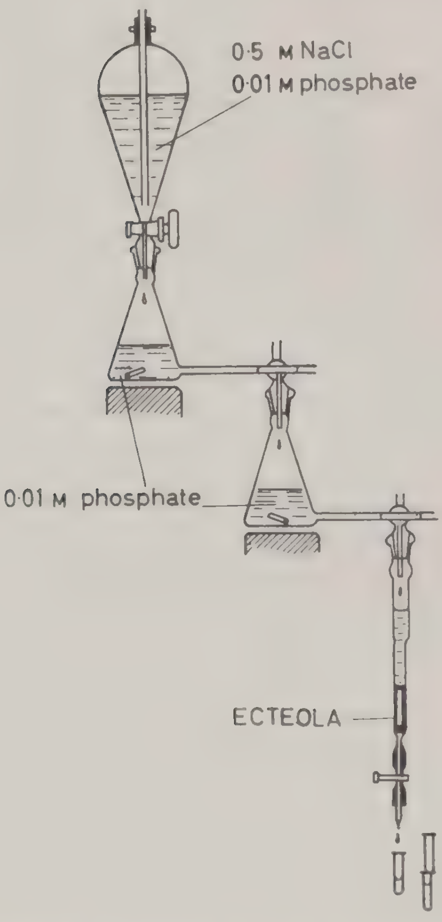

Figure 6. Apparatus used for gradient elution with two mixing chambers

(A. Bendich, H. B. Pahl, G. C. Korngold, H. S. Rosenkranz and J. R. Fresco: *J. Amer. chem. Soc.* 1958, **80**, p. 3952)

This expression is only valid if the volumes in the mixing chambers remain constant throughout the procedure. Volume changes can be avoided by using long (4–5 cm) heavy walled capillary tubing of internal diameter 2 mm. The eluent is collected at 2-hour intervals, with a flow rate of 2–5 ml. per hour, in an automatic fraction collector.

After the limit concentration of 0·5M sodium chloride is attained, very little additional nucleic acid is eluted with calf thymus deoxyribose nucleic acid if the sodium chloride concentration is raised to 2·0M. Also little more nucleic acid is eluted if the pH is raised by using ammonium hydroxide solution. However, if both the sodium chloride concentration and the pH are raised simultaneously, a large fraction of nucleic acid is released. In this way chromatography may be continued from 0·5M to 2·0M sodium chloride with a simultaneous rise of pH from 7·0 to 11·0. If the columns are finally washed with 0·5N sodium hydroxide a nearly quantitative recovery of nucleic acid may be obtained[31].

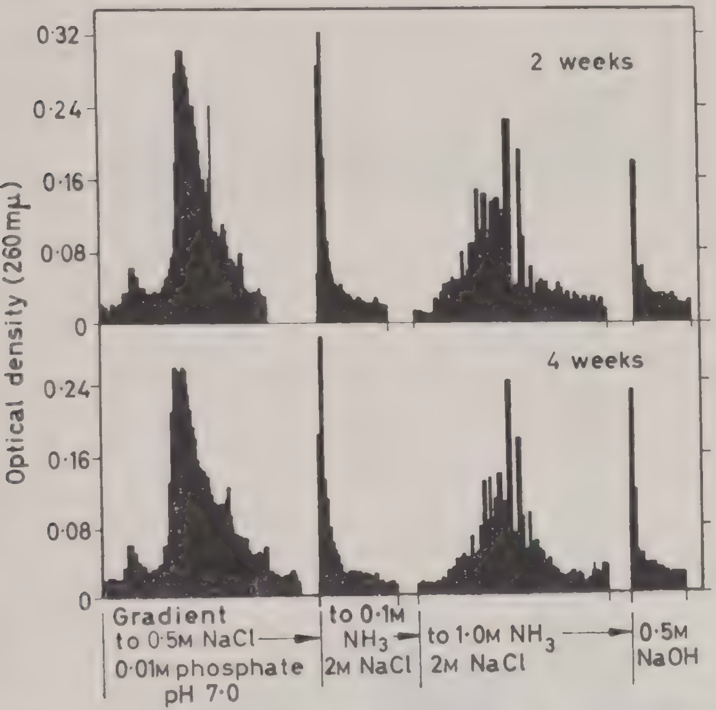

Figure 7. Chromatography on ECTEOLA of deoxypentose nucleic acid of regenerated rat liver 2 and 4 weeks after partial hepatectomy

(A. Bendich, H. B. Pahl, G. C. Korngold, H. S. Rosenkranz and J. R. Fresco: *J. Amer. chem. Soc.* 1958, **80**, p. 3954)

The extent to which the chromatographic profiles may be reproduced is shown in *Figure 7*, which gives independent chromatograms of deoxypentose nucleic acid isolated by the anionic detergent

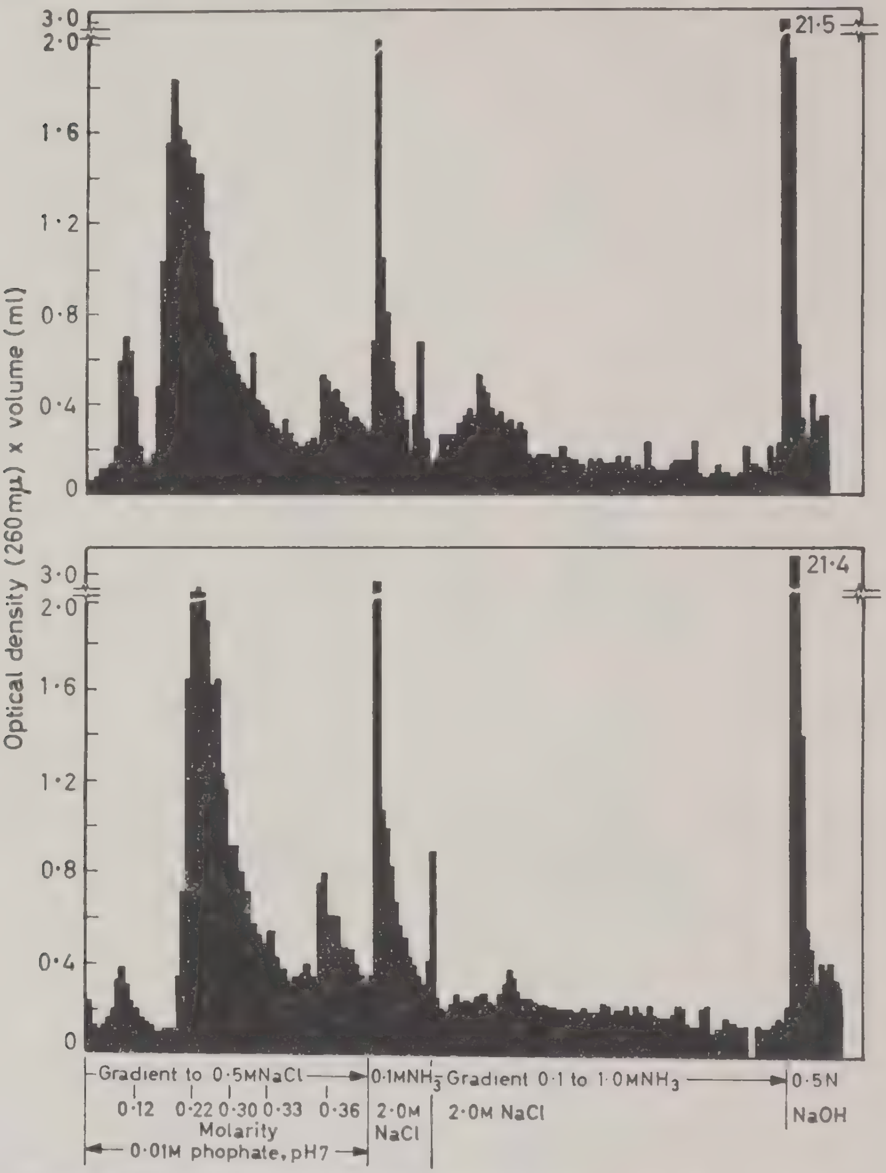

Figure 8. Chromatography on ECTEOLA of two samples of deoxypentose nucleic acid of human granulocyte prepared from the same starting material by two different methods. Upper chart prepared by the method of Kay, Simmons and Dounce. Lower chart prepared by extraction with sodium chloride and deproteinization by the chloroform-gel method (see Chapter 2). (A. Bendich, H. B. Pahl and S. M. Beiser: *Cold Spr. Harb. Symp. quant. Biol.* 1956, **21**, p. 34)

method from the nuclei of rat liver 2 and 4 weeks following partial hepatectomy. Most of the elution peaks in *Figure 7* are essentially superimposable. Such resolution can only be obtained if a flow rate of 3 to 5 ml. per hour is not exceeded. Any increase in the rate of elution will cause a decrease in the resolving power.

The chromatograms of deoxypentose nucleic acid from different sources vary and it would appear that a particular chromatogram is typical for the nucleic acid from each particular source. Bendich, Pahl and Beiser[28] have been able to differentiate between the chromatograms of deoxypentose nucleic acid from the following sources: calf thymus, human leukaemic leucocytes, *E. coli*, *B. cereus*, pneumococcus, T6r phage, T6r+ phage, and spleen, intestine, kidney and brain of the rat. A comparison of preparative methods has also been made by this procedure[28] and in *Figure 8* the chromatograms of two samples of the deoxypentose nucleic acid of human granulocyte are given. One preparation was made by the anionic detergent method and the other by the chloroform-gel method after extraction with sodium chloride. The similarity of the two chromatograms would suggest that the two methods yield almost identical products in contrast to the findings of Shooter and Butler[5] who from sedimentation studies concluded that the chloroform-gel method produced fractionation during extraction (see p. 40).

It is important that the basis of the chromatographic separation of polynucleotides on ECTEOLA columns should be established. This has been investigated by Bendich and collaborators[28, 30] from two points of view: (*1*) the purine and pyrimidine compositions of the various column fractions, and (*2*) the determination of the shape of the chromatographic profile as a function of the molecular size of the sample of deoxypentose nucleic acid. Analysis of fractions from calf thymus deoxyribose nucleic acid (*Table 1*) shows significant variations in the purine and pyrimidine composition[28, 31]. It would thus appear that the anion exchange method has effected a separation from the total sample of fractions of deoxyribose nucleic acid of varying composition. Examination of the values of the molar base composition in *Table 1* shows that there is no particular trend in the analysis of the fractions, in contrast to the observations of Chargaff, Crampton and Lipshitz[13] and of Brown and Watson[17] who found that the ratios of adenine to guanine and of thymine to cytosine increased with increase in the sodium chloride concentration of the eluting solution.

A possibly significant feature of the analytical data (*Table 1*) is the departure of the adenine to thymine and guanine to cytosine ratios from unity. As will be discussed later (Chapter 9) an

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important feature of the Watson–Crick model for deoxypentose nucleic acid is the specific hydrogen bonds between these pairs of bases and hitherto all analyses of deoxypentose nucleic acid have yielded ratios very close to unity (Chapter 4). It therefore would appear that the anion exchange procedure has a different basis of separation from the other methods used which have all produced fractions which show very little departure from the unity ratio for these base pairs. It would be interesting to know whether the binding of the polynucleotide to the anion exchanger is such as would produce breaking of the specific hydrogen bonds of the Watson–Crick model and thus yield single polynucleotide chains. If this were so, then the departure of the specific base ratio from unity could be explained. However, there is strong evidence that no significant change occurs in the structure of the nucleic acid on adsorption on to the ECTEOLA columns. Rechromatography of the column fractions yields a chromatogram in which the bulk of a given fraction appears at the same position in the elution profile as in the original fractionation. A definite structural change of the nucleic acid, as for example, when subject to heat treatment (*Figure 9a*) will cause a marked change in the profile. Of possibly more significance is the evidence that, on fractionation of pneumococcal transforming deoxypentose nucleic acid, the combined fractions from the column have almost the same biological activity as the original material[30, 38].

The amounts of deoxypentose nucleic acid present in each individual fraction are not sufficient for ultracentrifugal analysis, but Bendich, Pahl, Rosenkranz and Rosoff[30] have determined the distribution of sedimentation coefficients for certain regions of combined fractions for the deoxyribose nucleic acid of calf thymus. The chromatogram of calf thymus nucleic acid is shown in *Figure 9a* and in *Figure 9b* the sedimentation coefficient distribution of two fractions, eluted in Region I (*Figure 9a*) by (*1*) 0·16–0·24M sodium chloride and (*2*) 0·25–0·34M sodium chloride, are compared with the distribution for the original nucleic acid. The fractions show smaller weight average sedimentation coefficients, the value increasing with increasing concentration of the eluting solution in agreement with previous results[13, 17], and also exhibit a narrower distribution of sedimentation coefficient.

Useful information, having direct bearing on the relationship between molecular size and position of the fraction in the chromatogram, has arisen from studies on heated solutions of deoxyribose nucleic acid. The action of heat on solutions of deoxyribose nucleic acid (see Chapter 11, p. 256) at low ionic strengths, is to bring

Table 1. Purine–pyrimidine composition of column fractions of calf thymus deoxyribose nucleic acid

Fraction no.	*Fraction of original** %	*Composition of eluent*		*Molar base composition (Adenine = 1·00)†*			$\frac{\text{AD}}{\text{THY}}$	$\frac{\text{GU}}{\text{CY}}$	$\frac{\text{AD+GU}}{\text{THY+CY}}$	*Recovery* %‡
		NaCl molarity	NH_3 molarity	*Thymine*	*Guanine*	*Cytosine*				
Original	100·0			0·97	0·77	0·77	1·03	1·00	1·02	95
50–51, 53–54	3·5	0·20–0·29		0·91	0·61	0·81	1·10	0·75	0·94	91
55–57	3·4	0·29–0·31		0·88	0·64	0·84	1·14	0·76	0·95	89
59–64	2·6	0·34–0·39		0·94	0·71	0·82	1·06	0·87	0·97	90
146–161	3·2	2·0	Tris, pH 9·5§	0·97	0·73	0·83	1·03	0·89	0·96	92
197	3·3	2·0	0·10	0·88	0·67	0·74	1·14	0·91	1·03	89
199–200	3·6	2·0	0·10	0·95	0·64	0·74	1·05	0·87	0·96	98
206–211	3·1	2·0	0·10	1·01	0·75	0·79	0·99	0·95	0·97	95
236–237	3·3	2·0	0·25	0·98	0·63	0·74	1·02	0·85	0·95	100
239	5·4	2·0	0·35	0·95	0·68	0·72	1·05	0·95	1·01	95
242	6·9	2·0	0·45	0·98	0·78	0·78	1·02	1·00	1·01	99
244	3·1	2·0	0·53	0·94	0·86	0·90	1·06	0·96	1·01	104

*These values represent the amount of deoxyribose nucleic acid in each fraction as a percentage of the original, unfractionated material which was applied to the column.

†The average of six determinations except fraction number 146–161 where the guanine analysis was the average of only two analyses.

‡$\frac{\text{Total } \mu\text{moles of bases recovered}}{\text{Total } \mu\text{moles of phosphorus in hydrolysate}} \times 100.$

§Tris(hydroxymethyl)aminomethane buffer, 0·01M.

(A. Bendich, H. B. Pahl, G. C. Korngold, H. S. Rosenkranz and J. R. Fresco: *J. Amer. chem. Soc.* 1958, **80**, p. 3955)

about a decrease in the sedimentation coefficient, the viscosity and, if prolonged, in the molecular weight. The chromatograms of heated solutions of deoxyribose nucleic acid are shown in *Figure 9a* along with that of the unheated specimen. The chromatograms are clearly different from those of the untreated nucleic acid since the bulk of the nucleic acid heated in water was recovered in Region I,

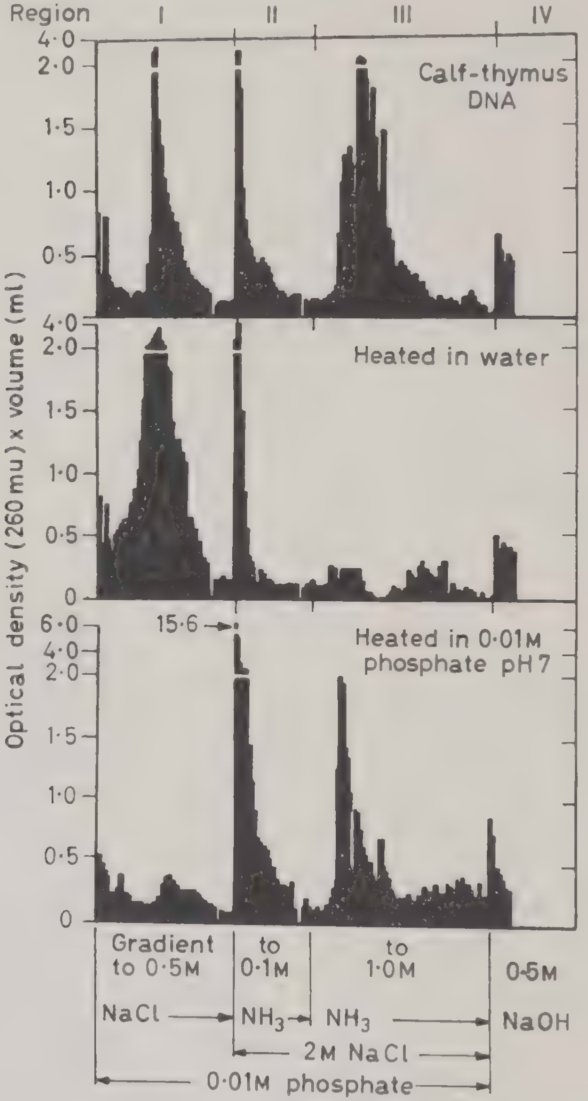

Figure 9a. Effect of heat on the chromatogram of deoxyribose nucleic acid of calf thymus

(A. Bendich, H. B. Pahl, H. S. Rosenkranz and M. Rosoff: *Symp. Soc. exp. Biol.* 1958, **12**, p. 38) whereas the heating in 0·01M phosphate buffer at pH 7·0 gave rise to a product which on chromatography showed hardly any of the first large peak characteristic of the unheated nucleic acid. Since the amount of nucleic acid in each column collection is directly proportional to the area below the curves (*Figure 9a*) it can be seen that the heating of the nucleic acid in the 0·01M phosphate solution results in an almost quantitative loss of the first peak and in a considerable loss of the nucleic acid in Region III (*i.e.*, in the 0·1–1·0M ammonia and 2M sodium chloride range). The lost fractions appeared in Region II presumably along with the material ordinarily eluted in this region. Since the chromatogram of the unheated material shows the largest amount of nucleic acid to be in Region III, it can be expected that this region contains much of the larger molecular weight material, which is almost quantitatively lost upon heating in water and which is subsequently recovered in the first peak, Region I. Since the effect of heat on deoxypentose nucleic acid may reduce the molecular size, it can be inferred that the fractions eluted in Region I are of small molecular size also. It thus transpires that the chromatographic

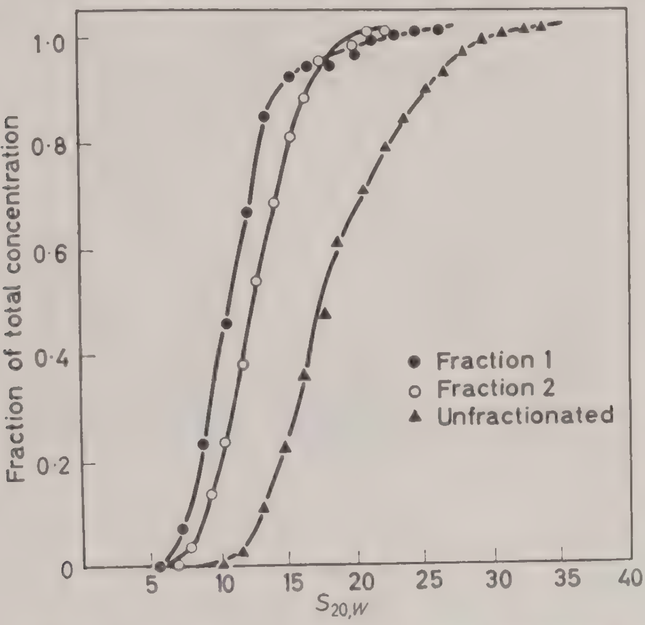

Figure 9b. Distribution of sedimentation coefficients of deoxyribose nucleic acid eluted in Region I (*Figure 9a*). Fraction 1 was eluted by 0·16–0·24M sodium chloride and Fraction 2 by 0·25–0·34M sodium chloride.
(*Symp. Soc. exp. Biol.* 1958, **12**, p. 36)

fractionation is dependent upon molecular size as well as upon composition.

The great interest surrounding any fractionation procedure centres around the possibility of isolating a fraction of deoxypentose nucleic acid which represents a particular genetical function. The only preparation of deoxypentose nucleic acid which possesses biological activity is the transforming deoxypentose nucleic acid from pneumococcus and this has been the subject of chromatographic studies in which the transforming activities of various fractions were also examined[28, 39]. The transforming deoxypentose nucleic acid from pneumococcus originally possesses four demonstrable genetic properties; these include transformations to penicillin, streptomycin and sulphonamide resistance, and mannitol utilization. The nucleic acid preparation, isolated from bacteria arising

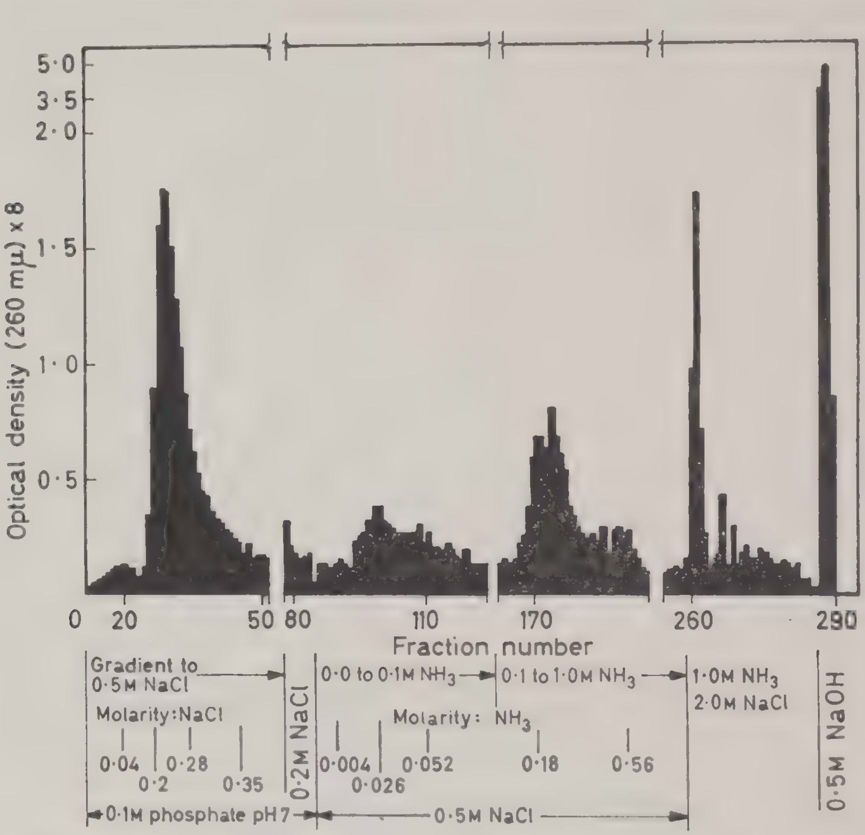

Figure 10. Chromatogram of transforming deoxypentose nucleic acid on ECTEOLA

(A. Bendich, H. B. Pahl and S. M. Beiser: *Cold Spr. Harb. Symp. quant. Biol.* 1956, **21**, p. 34) from a single clone, induces these heritable transformations at random essentially as single, independent events, although a significant number of the mutant cells show two properties, those of mannitol utilization and of streptomycin resistance which are acquired as related or connected events. It thus appears that the preparation of deoxypentose nucleic acid consists of at least five fractions, which may be identified as molecules, and each of four of these is associated with just one identifiable genetic property. In the fifth fraction two such properties appear to be linked together. The chromatogram of the transforming deoxypentose nucleic acid is shown in *Figure 10*, and the streptomycin transforming activity of some of the fractions is given in *Table 2*. The relative activities of the fractions vary greatly, some fractions possessing at least fifty times the activity of others. The most active fractions are approximately five times as active as the original material. These fractions were amongst those most strongly bound to the exchanger (ECTEOLA) and were eluted in the alkaline range; it is possible that the treatment with alkali may have

Table 2. Streptomycin transforming activities of chromatographic fractions of pneumococcal deoxypentose nucleic acid

Fraction no.	*Composition of eluent*		pH *of eluate*	*Relative transforming activity**		
	NaCl molarity	NH_3 molarity				
Original	—	—	7·0	1·0	1·0	1·0
25	0·17	—	7·0	0·7	0·8	0·7
27	0·19	—	7·0	0·2	0·4	0·2
29	0·21	—	7·0	0·5	0·6	0·4
32	0·25	—	7·0	0·4	0·1	0·04
35	0·28	—	7·0	1·2	0·8	1·9
40	0·32	—	7·0	0·9	0·7	1·2
67	0·45	—	7·0	0	0·1	0·06
78	0·47	—	7·0	0	0·1	0·03
99	0·50	0·02	8·4	1·9	1·8	2·0
173	0·50	0·22	9·5	—	5·1	4·2
190	0·50	0·56	10·4	1·1	1·0	—
260	2·00	1·00	11·0	—	5·1	4·8

* $\frac{\text{Transformants per } \mu\text{g fraction}}{\text{Transformants per } \mu\text{g original}}$. The activities listed in the three columns were determined on separate days.

(A. Bendich, H. B. Pahl and S. M. Beiser: *Cold Spr. Harb. Symp. quant. Biol.* 1956, **21,** p. 42) destroyed some of the activity so that these fractions may, in fact, initially have had an activity even greater than that observed. Similar results have been obtained by Ephrussi-Taylor[40] using a kieselguhr–histone column and by Lerman[22] who employed methylated bovine serum albumen to fractionate isotopically-labelled transforming deoxypentose nucleic acid.

From the data recorded in *Table 2*, it is evident that the ability to transform receptor cells to streptomycin resistance is associated with many of the fractions. Early fractions (no. 25) as well as late ones (no. 260) possess transforming activity. Many explanations could be put forward to explain this behaviour. It may be that the effect of molecular size heterogeneity is superimposed on the biological (chemical) heterogeneity and the early fractions, although smaller in size, still contain the specific transforming structure; alternatively more than one active site may be contained in each molecule and these may not be located in the same relative position in all molecules. Whatever the true explanation may be, it is evident that this method of fractionation is not, at present, producing fractions each of which can be associated with a particular discrete genetical activity.

THE HETEROGENEITY AND FRACTIONATION OF PENTOSE NUCLEIC ACID

The suggestion that samples of total pentose nucleic acid extracted from cells were heterogeneous was first made by Chantrenne[41] on the basis of the solubility of various metal salts of pentose nucleic acid. Chantrenne isolated two fractions which were subsequently re-examined for homogeneity by Ghuysen[42]. Bacher and Allen[43] also, on the basis of solubility determinations, investigated the heterogeneity of several pentose nucleic acids. These methods of fractionation, however, did not in general produce more than two fractions, one of low and one of high solubility. Ghuysen and Desreux[44, 45] fractionated yeast ribonucleic acid by fractional extraction at pH 3·5 and 5·5 and by fractional preparation with either acetic acid–acetone–water mixtures or methyl alcohol–sodium chloride–acetate buffer mixtures. In this way ten fractions were obtained all of which possessed the same purine–pyrimidine ratio. Many of the fractions which possessed identical molecular weights were found to have different solubilities.

Following the satisfactory use of ion-exchange resins for the fractionation of deoxypentose nucleic acid, Miura and Suzuki[46] and Bradley and Rich[47] employed this technique for the fractionation of pentose nucleic acids. The former authors employed Dowex-2

(Cl-form, 10 per cent cross-linkage) as the exchanger whereas the latter authors employed an ECTEOLA column prepared by Peterson and Sober.

The Dowex-2[46] column was washed repeatedly, first by acid, alkali and water and then thoroughly with 1M sodium chloride solution until the washings were free of ultra-violet absorbing material. The adsorption capacity of the resin was about 3·5 mg of pentose nucleic acid per 10 ml. of resin and the flow rate was kept at 0·2 ml./cm²/min. The nucleic acid was dissolved in 0·2M sodium chloride solution and the column washed with the same solution. The nucleic acid was then eluted by increasing the sodium chloride concentration stepwise from 0·5M to 5M and the concentration of nucleic acid in the eluate determined by measuring the absorption at 260 mμ.

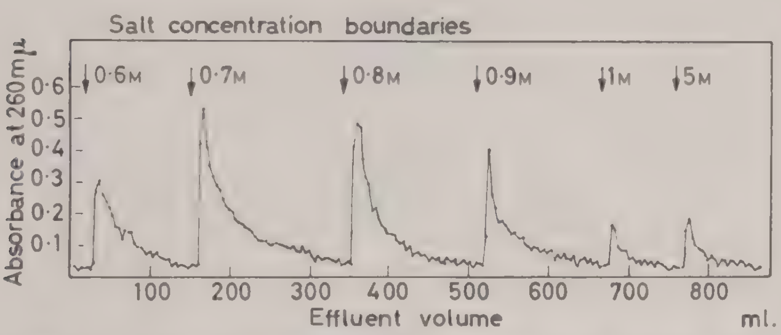

Figure 11. Discontinuous elution of pentose nucleic acid with sodium chloride solution from a Dowex-2 column

(K. Miura and K. Suzuki: *Biochim. biophys. Acta* 1956, **22,** p. 566)

A typical elution curve is shown in *Figure 11* where six fractions of yeast ribonucleic acid have been separated. The reproducibility of the method, as shown by rechromatography, was good. Molecular weights of the fractions were determined by the surface trough method, which is convenient because of the very small amounts of nucleic acid required, but is not very accurate. The molecular weight of the fractions was found to increase as the concentration of the eluting sodium chloride solution increased. Analysis for purines and pyrimidines showed no significant difference between the fractions in agreement with the observations of Ghuysen and Desreux[44, 45].

The experiments of Bradley and Rich[47] were mainly concerned with the variation of the sedimentation constant of the fractions obtained from various pentose nucleic acids after fractionation on ECTEOLA columns. The experimental technique employed was very similar to that of Bendich and collaborators described above[31]. In *Figure 12* are shown the chromatograms of seven different samples of pentose nucleic acid together with the mean sedimentation coefficient ($\bar{S}$) of the unfractionated nucleic acid. As the sedimentation coefficient increases the elution profile shifts towards higher salt concentrations. Profile (e) is of the pentose nucleic acid of tobacco

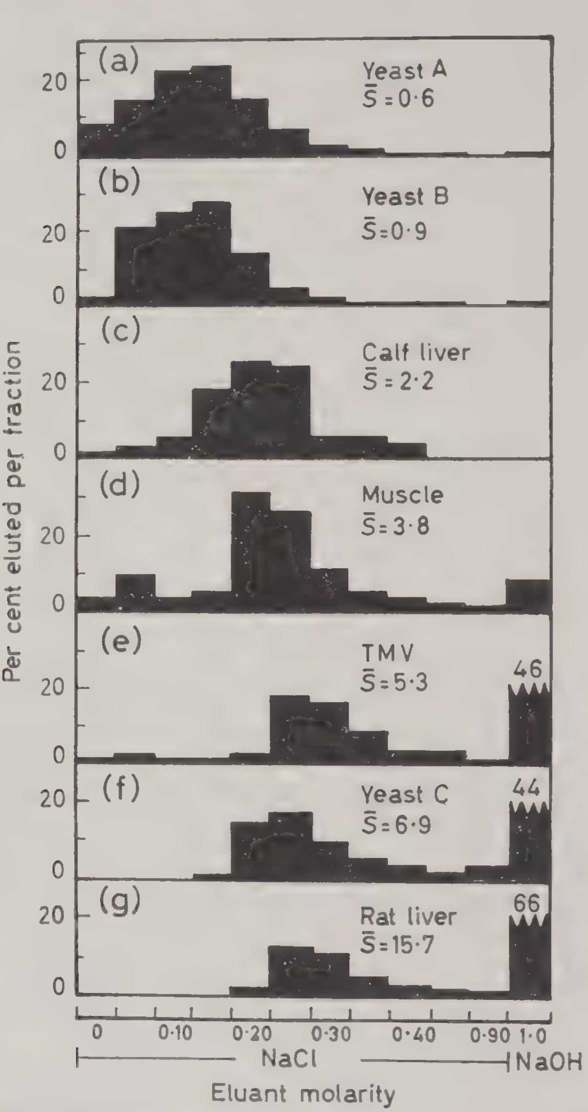

Figure 12. Chromatograms of different pentose nucleic acids on ECTEOLA
(D. F. Bradley and A. Rich: *J. Amer. chem. Soc.* 1956, **78**, p. 5899)

mosaic virus, which was studied by Bradley and Rich in the expectation that it might be more homogeneous than cellular nucleic acids. This was not found to be the case, but the heterogeneity may have, in part, arisen during isolation. In *Table 3* the mean sedimentation coefficients of the various fractions of the pentose nucleic acids are shown and in general the results are in agreement with the molecular weight measurements of Miura and Suzuki[46] in that the sedimentation constant increases as the concentration of the eluting sodium chloride solution increases.

Table 3. Mean sedimentation coefficients ($\overline{S} \times 10^{13}$) of pentose nucleic acids

Eluent NaCl molarity	*Yeast A*	*Yeast B*	*Calf liver*	*Muscle*	*Yeast C*
	Unfractionated				
	0·60	0·92	2·24	3·89	6·91
	Fractionated				
0·05	0·06	0·11	—	—	—
0·10	0·17	0·29	—	—	—
0·15	0·60	0·95	0·86	—	—
0·20	1·42	1·76	2·39	2·97	4·41
0·25	—	—	3·76	4·47	6·28
0·30	—	—	—	4·21	5·80
0·35	—	—	—	—	4·82

(From D. F. Bradley and A. Rich: *J. Amer. chem. Soc.* 1956, **78**, p. 5901)

These studies further emphasize the importance of the effect of the heterogeneity with respect to molecular size on the fractionation by ion exchange columns which was also observed with deoxypentose nucleic acids and which apparently tends to overshadow the influence of chemical heterogeneity.

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4

THE ISOLATION OF NUCLEIC ACID COMPONENTS AND THE COMPOSITION OF NUCLEIC ACIDS

THE ISOLATION OF PURINES, PYRIMIDINES, NUCLEOSIDES AND NUCLEOTIDES

The Hydrolysis of Nucleic Acids

THE hydrolysis of deoxypentose and pentose nucleic acids may yield a variety of products. These include purines and pyrimidines, pentose and deoxypentose according to the type of nucleic acid, the corresponding phosphorylated sugars, nucleosides, nucleotides and oligonucleotides (*i.e.* polynucleotides of very low molecular weight) as well as various degradation products formed by the further breakdown of these substances. The type of nucleic acid as well as the nature of the hydrolytic reagent is very important in determining the course of the degradation. The two types of nucleic acid show quite different behaviour on alkaline hydrolysis for reasons that will be discussed in Chapter 8. Pentose nucleic acids are readily hydrolysed to mononucleotides by treatment with 1M alkali at room temperature, whereas deoxypentose nucleic acids undergo little breakdown of a hydrolytic nature under these conditions; 4 hours at 100°C in this concentration of alkali are required in order to produce acid-soluble oligonucleotides[1]. In general the pentosides of both purines and pyrimidines are split with more difficulty than the deoxypentosides. However, it is characteristic of both types of nucleic acid that the purine pentoside or deoxypentoside linkage is more labile to acid than is the corresponding pyrimidine pentoside or deoxypentoside bond. The purines, adenine and guanine, are thus readily formed during acid hydrolysis of both nucleic acids, whereas the pyrimidines tend to remain as mononucleotides in the case of pentose nucleic acid and as nucleoside diphosphates with deoxypentose nucleic acid. The nucleic acids can only be hydrolysed to give the free pyrimidines, cytosine, uracil or thymine by drastic conditions.

Hydrolysis to purines and pyrimidines

Adenine and guanine are readily obtained from pentose nucleic acids by relatively mild acid hydrolysis. Treatment with alcoholic hydrochloric acid[2, 3] and with various concentrations of aqueous hydrochloric and sulphuric acids ranging from 0·4N to 6N[3–7] at 100–120°C for 1 to 2 hours have been used for both preparative[2, 8] and analytical purposes[3]. The main difficulty in the hydrolytic procedure, especially when used for analysis, is to obtain complete liberation of the purines without causing any deamination. The complete liberation of purine by treatment of the nucleic acid in 0·5N hydrochloric acid at 120°C for 2 hours has been questioned[4, 9], but judged by the recovery of adenine and guanine from the corresponding nucleotides by paper chromatography[3] and by precipitation as the silver salt[7], 1N acid at 100°C for 1 hour is quite satisfactory. These conditions have also been used for the analysis of pentose nucleic acids with almost complete recovery of the nitrogen-containing components as known purine and pyrimidine derivatives[6]. However, the isotope dilution method of determining purine concentration has indicated that about 7 per cent of the purine may be destroyed, probably by deamination, by this treatment.

While the above method yields adenine and guanine quantitatively from pentose nucleic acids, the pyrimidines, owing to the greater resistance of the glycosidic bond to acid hydrolysis, will remain as nucleotides or nucleosides. Although the pyrimidine components may be isolated[10, 11] or estimated analytically[12–14] after such treatment, it is more usual, for the purposes of analysis, to arrange the conditions so that the pyrimidines are liberated along with the purines.

The variety of different methods which have been employed from time to time in order to obtain a quantitative yield of all the bases, reflects the difficulty which has been experienced in perfecting a method. Cytosine and uracil may be prepared from yeast ribonucleic acid by hydrolysis with 25 per cent sulphuric acid at 175°C for 2 hours[15]. Under these conditions, however, there is deamination of cytosine to uracil. Other reagents which have been employed include 0·4–0·6N hydrochloric acid at 120°C[4], 98–100 per cent formic acid at 175°C[3], 20 per cent hydrochloric acid at 175°C[3], 12N perchloric acid[16], and trifluoroacetic acid at 155°C for 1 hour[17]. The results with 0·4–0·6N hydrochloric acid at 120°C show an increasing liberation of pyrimidines as the acid concentration is increased, but the yield of cytosine relative to uracil is low, indicating that deamination has occurred. Deamination of the cytosine is prevented by using concentrated formic acid, but the yield of uracil is very low and the method has been found unsatisfactory for the quantitative hydrolysis of uridylic acid[18]. Nearly quantitative yields of all the bases can be obtained by hydrolysis with 12N (70–72 per cent) perchloric acid at 100°C for 1 hour[16], although it would appear that the pyrimidines are not completely recovered. This method and, to a lesser extent, the method employing formic acid[3] have been extensively used for the analysis of nucleic acids. Recently, Dutta, Jones and Stacey[17] have recommended the use of trifluoroacetic acid at 155°C for 80 minutes; it causes no significant deamination of cytidylic acid and has the further advantage that it does not cause charring of the paper if the analysis is made by the method of paper chromatography.

Adenine and guanine are easily removed from deoxypentose nucleic acid by relatively mild treatment. The earliest methods employed 2 per cent acid at 100°C for 10 minutes and yielded in addition to the purines a rather ill-defined residue of high molecular weight which was named thymic acid[19] in the belief that it only contained thymine; later it was shown also to contain cytosine[20, 21]. By a more controlled method of preparation[22–24], pH 1·6 at 37°C for 24 hours or pH 2·8 at 100°C for 1 hour, a more reproducible material may be obtained which has been termed apurinic acid. For quantitative estimations of purines alcoholic hydrochloric acid has been used[3, 25]. By the use of stronger acid, the polynucleotide residue containing pyrimidines may be further degraded to yield thymine and cytosine deoxypentose diphosphoric acids and the corresponding mononucleotides.

For quantitative estimation of purines and pyrimidines, hydrolysis with hydrochloric acid, perchloric acid, formic acid and trifluoroacetic acid has been used under rather similar conditions to those used for pentose nucleic acids. Hydrochloric acid (6N) at 120°C for 2 hours gives a good yield of pyrimidines but causes some destruction of purines[25–27]. Better results are obtained with perchloric acid[16], a strength of either 7·5 or 12N may be used at 100°C for 1 hour. Loss of a small percentage of thymine may occur during hydrolysis with 12N perchloric acid, but this may be reduced by using not more than 15 μl. of perchloric acid per mg of deoxypentose nucleic acid. The most satisfactory results have been obtained, however, using formic acid. The conditions used[3] are 98–100 per cent formic acid at 175°C for 2 hours, but shorter periods of heating may be employed[28, 29]. Good results have also been obtained using weaker acid (88 per cent) at 175°C for 30 minutes[30], but whatever the concentration of acid employed, recoveries of guanine and hydroxymethylcytosine are improved by using a relatively large ratio of acid to nucleic acid (500 μl./mg) and by avoiding an oxidizing atmosphere. Recently trifluoroacetic acid at 155°C for 1 hour has been found to give very satisfactory quantitative results[17].

Hydrolysis to nucleotides

As has already been observed, pentose nucleic acid is readily hydrolysed to the mononucleotides[31]. The hydrolysis may be achieved with either 1N sodium hydroxide or 1N hydrochloric acid at room temperature. The latter procedure, however, is less advisable owing to the danger of breaking the purine pentoside linkage[11, 32, 33]. The main difficulty connected with this hydrolysis is the prevention of both further hydrolysis to the nucleosides and deamination. Amongst the methods that have been employed and which appear satisfactory are: pH 13–14 for 12 hours (overnight) at 30°C[18], 0·5N sodium hydroxide at 20–22°C for 18 hours[34], concentrated ammonium hydroxide at 45°C for 8 days[34] (under other conditions ammonia may produce nucleosides), 0·3N potassium or sodium hydroxide at 37°C for 18 hours[35–37] (higher concentrations of alkali, *e.g.* 1N potassium hydroxide may cause deamination of cytidylic acid). The nucleotides produced by these procedures are all mixtures of the nucleoside-2′- and -3′-phosphates (see below).

Chemical hydrolysis is not a satisfactory method for the preparation of mononucleotides from deoxypentose nucleic acids on account of the lability of the glycosidic linkage of the purine deoxypentose nucleotides to acid and the resistance of this nucleic acid to alkaline hydrolysis. Under conditions that will produce alkaline hydrolysis, *viz.* 1N sodium hydroxide at 100°C for 4 hours, oligonucleotides are formed and almost complete deamination of the purines and cytosine occurs[1]. However, mild acid hydrolysis will produce a mixture of the pyrimidine-5′-phosphates and the -3′,5′-diphosphates[38–44].

Enzymic hydrolysis of the nucleic acids may also be used for the preparation of nucleotides. The enzymic hydrolysis of pentose nucleic acids by ribonuclease, however, is not complete[45]. This is because ribonuclease does not hydrolyse all the bonds between the component nucleotides of pentose nucleic acid, but only those between the 3′-pyrimidine nucleoside phosphoryl groups and the 5′-hydroxy groups of adjacent purine or pyrimidine nucleotides[46–48]. The bonds between adjacent purine nucleotides and between a 5′-pyrimidine phosphoryl group and a 3′-hydroxy group of the adjacent purine or pyrimidine nucleotide are therefore not hydrolysed. The final products of the ribonuclease action are therefore 3′-uridylic acid and 3′-cytidylic acid and a mixture of oligonucleotides. The nucleoside-5′-phosphates may also be obtained from pentose nucleic acids, but for reasons which will be discussed in Chapter 8, these nucleotides cannot be obtained by chemical hydrolysis. However, snake venom phosphodiesterase, when freed of 5′-monoesterase activity, produces a mixture of the four 5′-nucleotides, of pyrimidine-2′,5′- and -3′,5′-diphosphomononucleotides and purine nucleosides[49–51] from which the 5′-nucleotides may be isolated by ion-exchange chromatography.

The deoxypentose nucleotides may also all be prepared by enzymic hydrolysis. The deoxypentose nucleoside-5′-phosphates[52, 53] may be isolated in good yield by ion-exchange chromatography after digestion of deoxypentose nucleic acid with the deoxyribonuclease of calf pancreas. The preparation of the deoxypentose nucleoside-3′-phosphates has been recently described using the deoxyribonuclease isolated either from *Micrococcus pyogenes*[53a] or from calf spleen[53b].

Hydrolysis to nucleosides

The pentose nucleosides may be prepared from pentose nucleic acid using more drastic hydrolysis conditions than those used for the preparation of the nucleotides. Levene and Jacobs[53c] used concentrated ammonium hydroxide under pressure at 175–180°C for 3·5 hours to effect the hydrolysis, whereas Bredereck, Martini and Richter[53d] employed aqueous pyridine. Modifications have been made to both methods to improve the yield[53e–g]. The nucleosides may also be obtained from the individual nucleotides by either chemical hydrolysis[53g, h], the pyridine hydrolysis being generally preferred, or by the action of phosphatases[53i–k].

The stability of the phosphodiester linkage in deoxypentose nucleic acids and the instability of the glycosidic linkage in the purine deoxypentose nucleosides preclude the use of acid and alkali in the hydrolysis to deoxypentose nucleosides. The preparation must therefore be by enzymic hydrolysis. Anderson, Dekker and Todd[61] have described a method whereby the deoxypentose nucleic acid is first hydrolysed by deoxyribonuclease, and without isolation, the deoxypentose nucleotides are then dephosphorylated by intestinal phosphatase in the presence of silver ions to inhibit adenine deoxypentoside deaminase.

The Separation of the Hydrolysis Products by Ion-Exchange Chromatography

The separation, by ion-exchange chromatography, of the purines, pyrimidines, nucleosides and nucleotides formed by the hydrolysis of nucleic acids may be used not only as a method of analysis but also for the preparation of these compounds. It has thus been used quite extensively in recent years for the preparation of nucleosides and nucleotides which cannot be prepared by other methods and one of the most important successes of the method has been the isolation of the isomeric pentose nucleotides with the phosphate group on the 2′- and 3′- position of the pentose. The method has been developed very largely by Cohn and his collaborators[54, 55].

The general methods of ion-exchange chromatography have been extensively described elsewhere[56] and the standard techniques are generally employed for the nucleic acid degradation products. The analysis of the eluent is made by determining the extent of the absorption of the solution at 260 mμ. The ion-exchange resins most generally employed have been the strong base anion exchangers, Dowex-1, Dowex-2 and Amberlite-IRA-400; the strong acid cation exchangers, Dowex-50 and Amberlite-IR-120; and the weak acid cation exchanger, Amberlite-IRC-50.

The free purines and pyrimidines and the nucleosides may contain various ionizing groups. Neglecting the sugar hydroxyl groups which are not ionized except in very strong alkali, these are: amino groups in adenine, guanine and cytosine and the —NH—CO— dissociation in guanine, uracil and thymine. These have pK_a' values (see Chapter 7) in the range 3·2–4·5 and 9·5–10·0 respectively. There is also a weak acid dissociation in adenine having a pK_a' value of 9·8 which most probably represents the dissociation of —NH— to $—\bar{N}—$ in the imidazole ring.

With the nucleotides, in addition to the above dissociations, there are also present the primary and secondary phosphoric acid dissociations with pK_a' values of about 1·0 and 6·5 respectively. With this variety of charged groups elution may be carried out either by increasing the concentration of the competing ion and maintaining the pH, and hence the charge on the ions constant or more generally by changing the pH, so that the charge on the adsorbed ion is reduced or reversed.

The presence of the phosphoric acid groups on the nucleotides produces a marked difference in the ion-exchange behaviour of these substances compared with the purines, pyrimidines and nucleosides. The behaviour of these two groups of substances will therefore be considered separately.

In acid solution, the amino groups of guanine, adenine and cytosine and of the corresponding nucleosides are ionized as cations, the pK_a' values increasing in that order (see *Tables 8* and *9*, Chapter 7). Uracil and thymine and the corresponding nucleosides have no amino groups, and are therefore not adsorbed to any appreciable extent by cation exchangers in the hydrogen form. *Figure 13* shows the separation of an artificial mixture of four bases from yeast ribonucleic acid by cation exchange on a Dowex-50 column (H^+ form). Elution was carried out by raising the concentration of the competing ion without change of charge[54, 55, 57]. Thymine is inseparable from uracil in this system as are thymidine and uridine, since they are not cations and are therefore not adsorbed. Cytidine precedes cytosine and 5-methylcytosine follows it closely[58]. Elution by increasing the pH of the solution has not been used for separating mixtures of purines and pyrimidines although Reichard and Estborn[59] have used this method for the separation of isotopically-labelled deoxypentose nucleosides. In general, however, this method is not to be recommended for the preparation of the nucleosides, since extensive hydrolysis of the purine nucleosides, due to the lability of the purine glycosidic linkage, occurs on the columns[57, 60, 61].

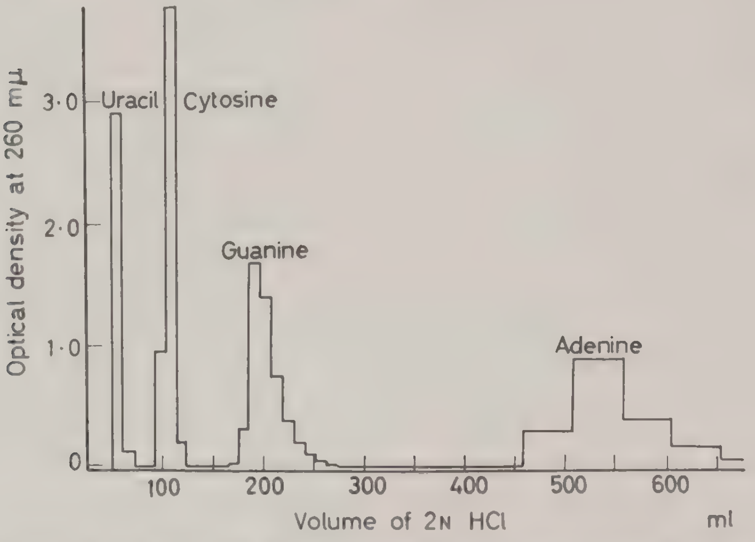

Figure 13. Separation of a mixture of adenine, guanine, cytosine and uracil by a cation exchange column (Dowex-50–H^+)
(W. E. Cohn: *Science* 1949, **109**, p. 377)

The anionic properties of guanine, uracil and thymine, and of the corresponding nucleosides, reside in the —NH—CO— dissociation. In adenine there is the dissociation in the imidazole ring which cannot occur in adenosine owing to the removal of the acid hydrogen atom in the formation of the glycosidic linkage. Cytosine and cytidine possess no anionic properties. Separation of the five purines and pyrimidines is shown in *Figure 14.* The positions of the elution peaks of the nucleosides tend to overlap those of the corresponding bases as is shown in *Figure 15.* Adenosine is an exception; the loss of the hydrogen atom from the imidazole ring results in the complete loss of acidic properties, making the exchange properties of adenosine similar to those of cytidine and cytosine from which it is very difficult to effect a separation (*Figure 15*). However, Andersen, Dekker and Todd[61], in a method suitable for the large scale preparation of deoxypentose nucleosides, have successfully isolated the adenine and cytosine deoxypentose nucleosides which were eluted together from a Dowex-2 column by ammonium formate buffer (pH 9·8) by crystallizing out the adenine deoxypentose nucleoside monohydrate and purifying the supernatant on the weak acid cation exchanger, Amberlite-IRC-50, and have thus obtained the cytosine nucleoside.

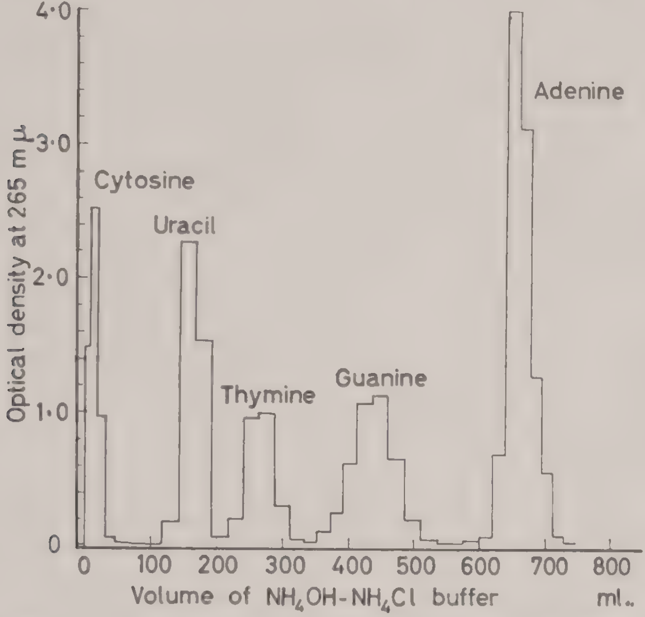

Figure 14. Separation of a mixture of adenine, guanine, uracil, thymine and cytosine by an anion exchange column (Dowex-1–chloride)
(W. E. Cohn: *Science* 1949, **109**, p. 377)

The nucleotides differ from the purines, pyrimidines and nucleosides in ion-exchange characteristics owing to the presence of the phosphoric acid group which tends to dominate the exchange behaviour. Cytidylic, adenylic and guanylic acids, owing to the presence of the amino and phosphate groups, remain anionic down to quite low pH values; the isoelectric points of these ampholytes are between pH 1 and 2. As is shown in *Figure 16*, in the range

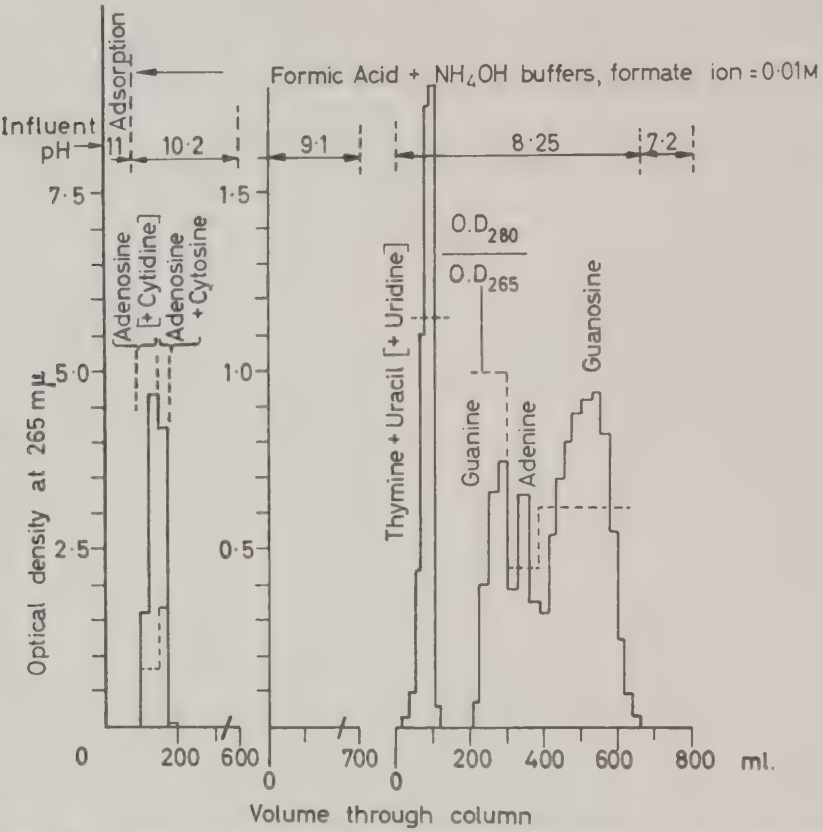

Figure 15. Separation of purines, pyrimidines, nucleosides and nucleotides by anion exchange (Dowex-1–formate)
(From W. E. Cohn: *J. Amer. chem. Soc.* 1950, **72**, p. 1474)

pH 5·5–2·0, the net negative charge decreases as the pH is lowered owing to the ionization of the amino group, and as this group possesses different pK_a' values in the different nucleotides, the net charge on the individual nucleotides at a given pH value is different. This difference in net charge forms the basis of the ion-exchange separation. Uridylic acid (and also thymine deoxypentose nucleotide) possesses no amino group and does not attain a positive charge. The change of negative charge shown in *Figure 16* below pH 2·0 is due to the titration of the ionized primary phosphoric acid dissociation.

The order of elution predicted by a consideration of net charge is subject to modification owing to the greater attraction of the exchanger for purines over pyrimidines. Thus in order to explain the observed separations (*Figure 17*) it is necessary to assume that the purine compounds are adsorbed on to the exchanger about three times as strongly as the pyrimidines[60].

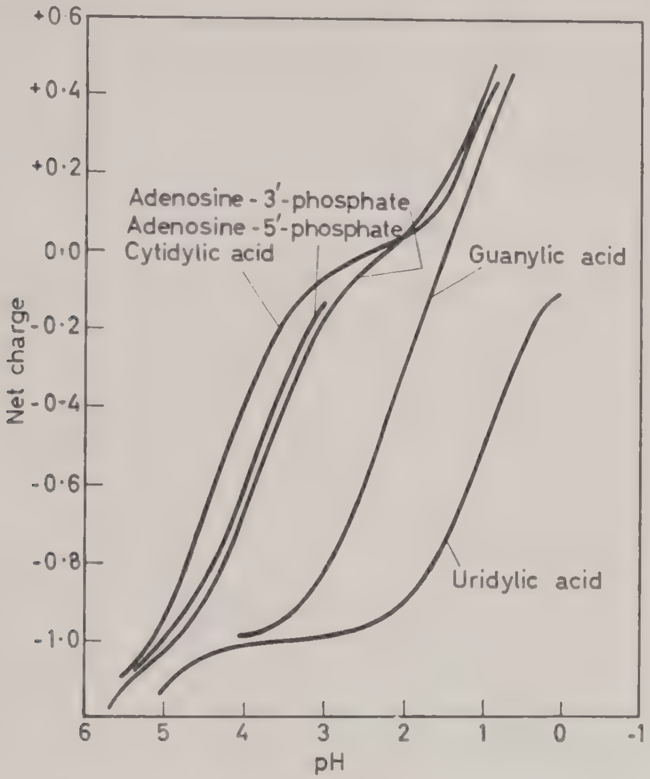

Figure 16. Charge per molecule of ribonucleotide as a function of pH

(W. E. Cohn : *J. cell. comp. Physiol.* 1951, **38,** Supplement 1, p. 24)

The separation of the four pentose nucleotides (mixed -2′- and -3′-phosphates) has been fully described by Cohn and Khym[62] and a typical chromatogram for elution by dilute hydrochloric acid at pH 2·5 is shown in *Figure 18.*

The anion exchange resin (Dowex-1 or -2, Amberlite-IRA-400 or -410, 200–400 mesh, 8–10 per cent divinylbenzene) is used in the chloride form, and the column is constructed by slurrying the appropriate amount of exchanger into a glass tube containing a coarse sintered glass disc[56]. The minimum column height should be 10 cm and the minimum volume of resin should be 20 ml./g of mixed nucleotides. A greater height and volume will improve the degree of separation, but will slow the operation. The resin is prepared by flowing through 10 column volumes (CV) of 1N sodium hydroxide, 2 CV of 1M ammonium chloride, 10 CV of 1N hydrochloric acid and 2 CV of water (to give a pH in the effluent of over 3). After the alkaline hydrolysis of the pentose nucleic acid (see above), the solution is titrated to pH 5 with a sulphonic acid–polystyrene ion-exchanger[63] in the hydrogen form (Dowex-50 or Amberlite-IR-120) and the exchanger removed by filtration or decantation. The exchanger is washed and the washings added to the filtrate.

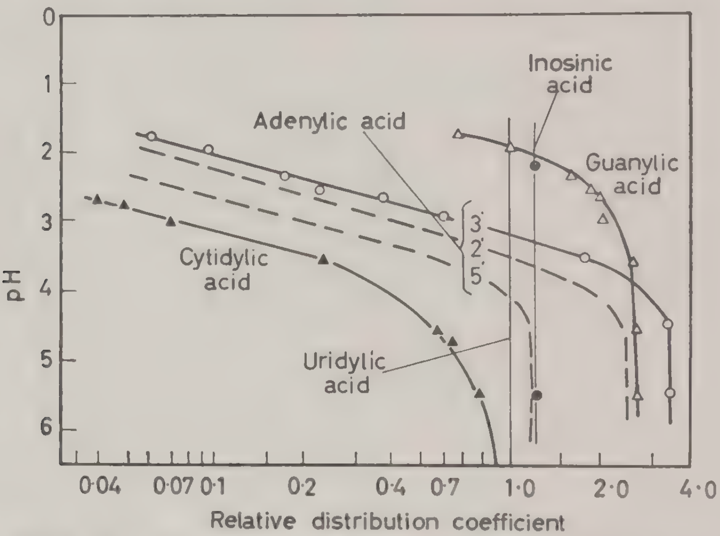

Figure 17. Observed relative distribution coefficients (positions of elution peaks) of ribonucleotides as functions of pH (W. E. Cohn: *The Nucleic Acids* Vol. 1 (Ed. E. Chargaff and J. N. Davidson): Academic Press, New York, 1955, p. 223)

The filtrate is brought to a pH of greater than 8 by the addition of ammonia, diluted to a total anion (including nucleotides) concentration of 0·01M and adsorbed on the exchanger at a flow rate of not more than 3 ml./cm^2/min. The column is washed with 2 CV of water and 10–20 CV of 0·01M ammonium chloride (until pH of effluent drops to below 7) to remove nucleosides, purines and pyrimidines. If inorganic phosphate is present in the hydrolysate 0·04M ammonium chloride (10 CV) should be used, followed by 2 CV of water[64].

The nucleotides are eluted with dilute hydrochloric acid (0·002N), the spectrophotometric absorption at 260 mμ being used to follow the course of the elution. In the first 10 CV the cytidylic acids are removed, followed by the adenylic acids (which appear at about 30 CV). When the optical density of the eluate has again fallen to less than 0·01 about 100 CV of 0·005N hydrochloric acid are used to elute the uridylic acids and the guanylic acid in that order. The separation may also be achieved with 0·1M formic acid on a formate column (substitute formate for chloride in the preliminary steps) or with 0·3M acetic acid on an acetate column (substitute acetate for chloride in the preliminary steps)[62].

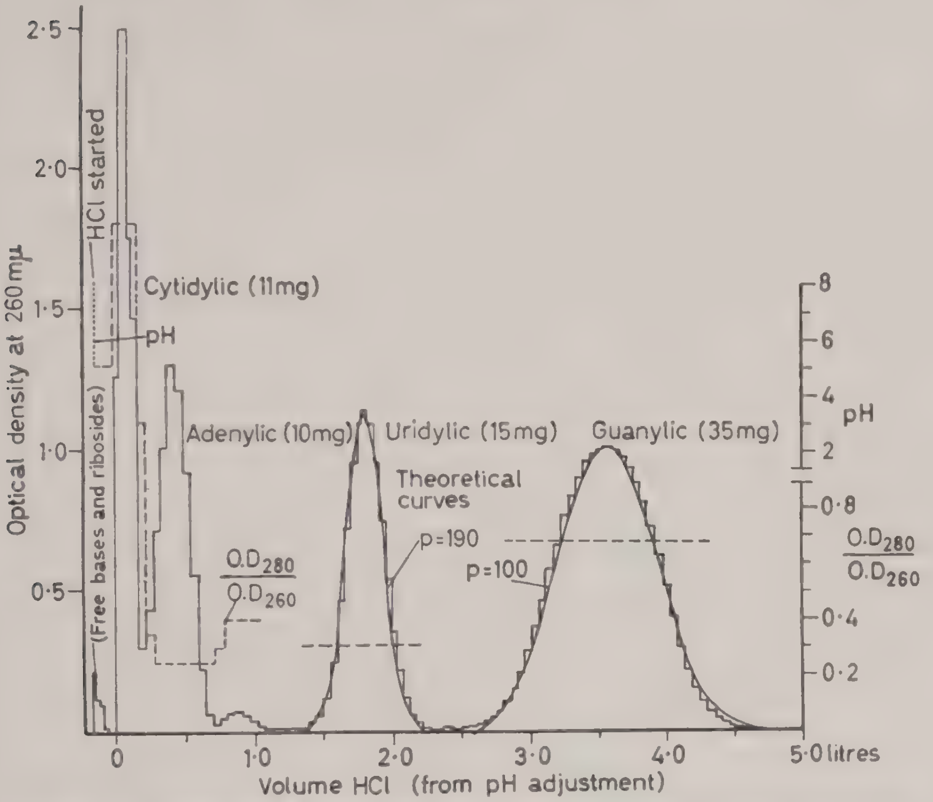

Figure 18. Separation of ribonucleotides by anion exchange (Dowex-2–chloride) Continuous line represents the calculated theoretical curve (W. E. Cohn: *J. Amer. chem. Soc.* 1950, **72**, p. 1476)

The separation of the deoxypentose nucleotides (-5′-phosphates) after enzymic digestion of deoxypentose nucleic acid was first described by Volkin, Khym and Cohn[52] and a typical chromatogram is shown in *Figure 19*. The separation[52, 65–67] follows a similar pattern to that for the pentose nucleic acids with thymine deoxypentose nucleotide (thymidylic acid) taking the place of uridylic acid. A full description of the procedure is given by Cohn, Volkin and Khym[68].

A 0·1–1·0 per cent solution of deoxypentose nucleic acid is heated to 100°C in a water bath for 10 minutes to remove any phosphomonoesterase[67], the solution is then made 0·03M with respect to magnesium sulphate (Mg : P = 1 : 10) and 0·02 per cent in crystalline deoxyribonuclease[68] and incubated at 37°C. The pH should be maintained at a value between 6 and 8 during the incubation by the dropwise addition of dilute sodium hydroxide. After 1–2 hours a diesterase preparation from rattlesnake venom[69] is added and the pH adjusted to a value between 8 and 9. The hydrolysis is complete in 5–6 hours during the course of which the

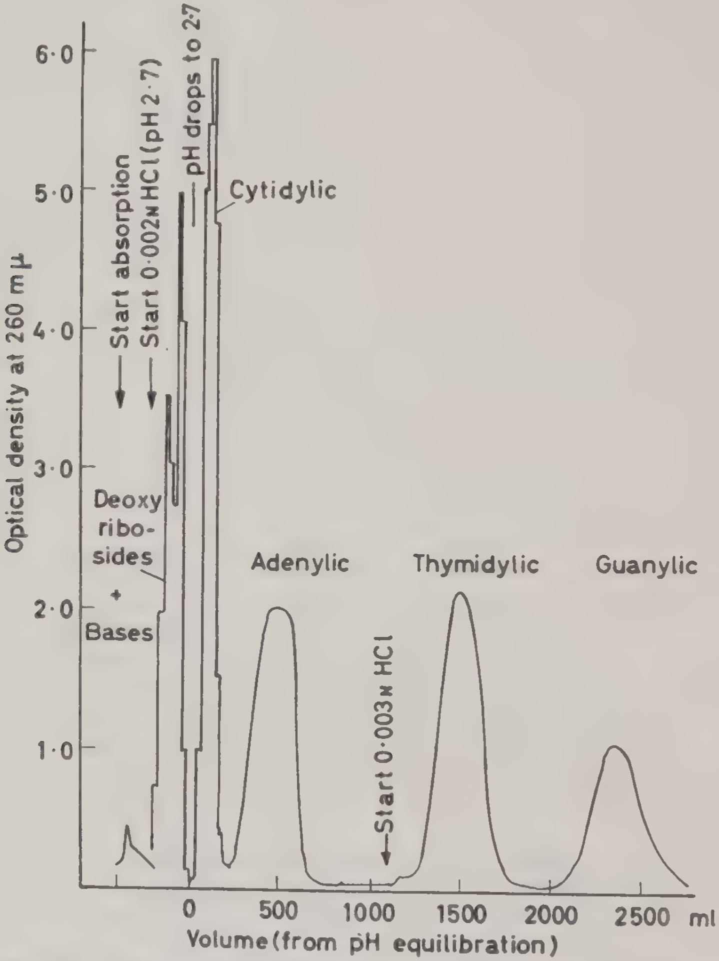

Figure 19. Separation of deoxypentose nucleotides by anion exchange (Dowex-1–chloride)
(E. Volkin, J. X. Khym and W. E. Cohn: *J. Amer. chem. Soc.* 1951, **73**, p. 1534)

solution is titrated with dilute sodium hydroxide to maintain the pH constant. After the hydrolysis is complete ammonium hydroxide is added to give a concentration of 0·1M, the solution diluted to give a total anion concentration (including nucleotides) of less than 0·01M and adsorbed on the exchanger. The chromatography is carried out as for the pentose nucleotides on a column in the chloride form. After adsorption on the column and washing as before, deoxycytidylic acid is eluted with about 50 CV of 0·001N and deoxyadenylic acid with 40 CV of 0·002N hydrochloric acid. Thymidylic acid is eluted with 30 CV of 0·003N and deoxyguanylic acid with 0·005N hydrochloric acid (about 70 CV). Formate or acetate columns may also be used.

One of the most important results of the ion-exchange chromatography of the alkaline hydrolysates of pentose nucleic acids has been the discovery and isolation of the isomeric nucleoside-2′- and -3′-phosphates[60, 70, 71]. The -5′-phosphates are not obtained by chemical hydrolysis. The discovery of the existence of the nucleoside-2′- and -3′-phosphates has considerably aided the elucidation of the structure of the pentose nucleic acids and our understanding of the mechanism of the alkaline hydrolysis of pentose nucleic acids (see Chapter 8).

The dissociation constants of the isomeric adenylic and cytidylic acids have been determined (see *Table 9*, Chapter 7) and the pK'_a values of the amino groups and of the secondary phosphoric acid dissociations decrease in the order 5′-, 2′-, 3′-. Since the isomers appear in this order on elution under the conditions necessary for separation, it would appear, since other factors will be almost constant in the isomers, that the dissociation of these groups is responsible for the separation[55].

Adenosine-2′-phosphate (adenylic acid *a*) and adenosine-3′-phosphate (adenylic acid *b*) may be separated by using a chloride[60, 70], formate[54] or acetate system (*Figure 20*). The formate system has frequently been preferred, partly because of the ease of obtaining buffered solutions of pH 3–4 and partly because of the ease with which formic acid and its ammonium salt can be volatilized. However, dilute hydrochloric acid has the advantage that if concentration is to be carried out by re-adsorption and elution no dilution is necessary, whereas the concentrated formate and acetate solutions require dilution before re-adsorption[60, 72]. The adenylic acid fraction from an initial separation is re-adsorbed on to an identical column. Two mg of adenylic acid per ml. of column are advisable and the total anion concentration (including nucleotides) should not be greater than 0·01M. The column is washed as before, and the isomeric adenylic acids eluted with 150 CV of 0·02N hydrochloric acid[62]. Adenosine-2′-phosphate appears after about 45 CV, with a peak at 70 CV and adenosine-3′-phosphate reaches a peak at about 120 CV. Cytidine-2′- and -3′-phosphates are separated in a similar manner using 0·001M hydrochloric acid[62] or a formate or acetate system[50, 54, 60, 70]; uridine-2′- and -3′-phosphates are separated on a formate column using 0·05M ammonium formate and 0·00125M formic acid[50, 54, 60, 70], and the guanosine-2′- and -3′-phosphates separated on a chloride column with 0·005N hydrochloric acid[54, 60, 62, 70]. The separated isomers are concentrated from the dilute solution by re-adsorption on smaller columns and stripped in small volumes of 0·1N hydrochloric acid[62].

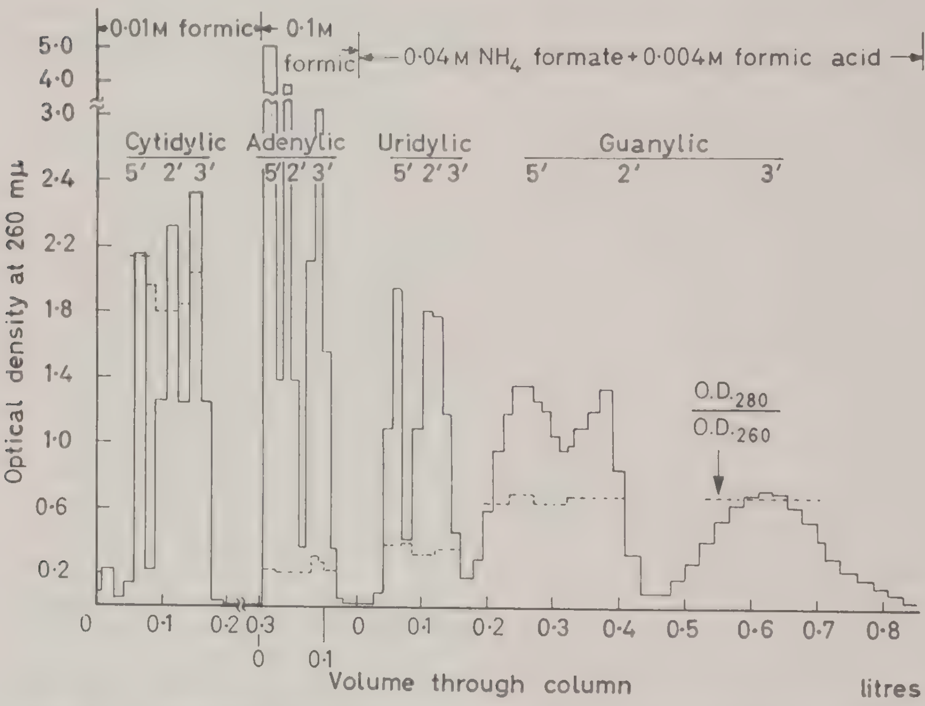

Figure 20. Separation of the isomeric -2′-, -3′- and -5′-ribonucleotides by anion exchange (Dowex-1–formate)

(W. E. Cohn: *The Nucleic Acids* Vol. 1 (Ed. E. Chargaff and J. N. Davidson): Academic Press, New York, 1955, p. 230)

The ion-exchange method of separation may also be applied to nucleoside diphosphates, oligonucleotides, and as described in Chapter 3 to nucleic acids. Complexing agents have also been used to aid the separation. The most useful of these is boric acid, generally present in solution as the tetraborate ($K_2B_4O_7$). By using a borate system it is possible to increase markedly the adsorption of the nucleosides[55, 73]. The separation of the nucleotides and of the isomeric nucleotides may also be carried out in borate systems with some improvement of resolution[74–76].

The Separation of Hydrolysis Products by Paper Chromatography

Since the first demonstration of the separation of the components of nucleic acids by paper chromatography by Vischer and Chargaff[77] and by Hotchkiss[4], progress of the method has been rapid and its use as a quantitative method of analysis has become general. It has the advantage over the ion-exchange method of being more rapid, although it is less applicable to the isolation of materials in quantity. The presence in the nucleic acid components of acid–base groups which facilitate their adsorption has already been discussed. The main experimental difficulty which has had to be overcome in applying the paper chromatographic technique has been the determination of the positions of the spots on the paper, as the whole success of the method depends upon the identification of the spots by a sensitive and convenient method.

The first methods relied upon the formation of mercury complexes with the nucleic acid components, which were made visible by converting the mercury to its sulphide[77] and upon making arbitrary cuts in the paper and estimating the concentration by elution and spectrophotometry of the eluent[4]. Neither of these methods was very satisfactory, the former being cumbersome and relatively insensitive and the latter lacking in resolving power. Marked improvement came with the use of ultra-violet lamps having a high emission in the range where the absorption by nucleic acid components is a maximum. The positions of the nucleic acid components are then observed as dark regions against the background fluorescence of the filter paper[78–80]. A low pressure mercury arc lamp (which may emit as much as 80 per cent of its radiation as the resonance line of 254 mμ) together with a quartz filter is used. An alternative procedure which is rather more laborious, but provides a permanent, natural size record of the chromatograph is obtained by taking photographic contact prints of the chromatogram in ultra-violet light[81, 82]. The light used is filtered, a cobalt and nickel sulphate solution and chlorine gas being used to isolate the 254 mμ and 265 mμ lines from a high pressure mercury lamp[12]. Slightly greater sensitivity has been claimed by utilizing the 257 mμ and 275 mμ lines of a cadmium arc[83]. In the developed print the positions of the substances appear as white areas on a dark background ; these areas may then be traced on the chromatogram itself.

The photographic method may also be used for an extremely sensitive procedure for detecting spots of substances, such as guanine and its derivatives, which fluoresce under certain circumstances[84, 85]. This method has been used to detect the presence of small amounts of 8-azaguanylic acid in various specimens of pentose nucleic acid from organisms treated with 8-azaguanine[86, 87]. This effect may be recorded photographically by inserting a sheet of cellulose nitrate between the chromatogram and the photographic paper.

Other detection methods, which are suitable for nucleotides and other phosphoric acid esters, are the spraying of the paper chromatogram with acid molybdate solution, partial hydrolysis of the ester by heat, light, or enzymes, and reduction of the resulting phosphomolybdate complex to a blue-coloured compound[88, 89]. Methods which avoid the need for hydrolysis of nucleotides include fixation with uranium[90] and ferric ions[91]. The various sugar groups may be used to detect the nucleosides[92]. The pentose nucleosides may be detected by the oxidation of the pentose with periodate to give the dialdehyde, which may then be detected by Schiff's reagent. Deoxypentose nucleosides may be detected by the Dische diphenylamine reaction or by the Feulgen reaction.

The chromatographic procedure has been described by Wyatt[93] and by Markham[94]. Descending chromatography is generally employed so that the solvent may run off the paper although the ascending method is convenient for small two-dimensional chromatograms. Whatman No. 1 paper is suitable for most purposes but the thicker No. 3 MM is to be preferred for preparative work. For quantitative analysis about 50 μg of each purine or pyrimidine or its equivalent should be present in each spot although smaller amounts have been used successfully. The solution is best applied as a spot of 10–15 μl. Blanks should be taken from parallel lanes at the same level as the experimental spots and both are extracted in 5 ml. of 0·1 N hydrochloric acid overnight before the concentrations are determined spectrophotometrically.

A great variety of solvent systems has been employed, even though the choice of solvent is somewhat restricted by the fact that substances which absorb in the ultra-violet cannot be used. Most systems, however, are based on the aliphatic alcohols, which may be used in a neutral, acid or alkaline state. An important factor is the relative amount of water present in the solvent; for example, by altering the water content of propanol–water mixtures, the relative position of adenine and adenylic acid in the chromatogram may be reversed[82]. For this reason, a fixed concentration of water should be employed, generally just below saturation, rather than a saturated solution, so that the water concentration is independent of temperature. Other factors which affect the separation are the pH and ionic strength. Ionization of the nucleic acid components will alter the partition in favour of the aqueous phase; control of the pH is thus important. The main influence of altering the salt concentration is on the mutual solubility of water and the organic solvent, but the solubility of the solute may also be affected and its adsorption increased. Aqueous *n*-butanol, either with or without added ammonia, was one of the first solvents used for the separation of nucleic acid components[4, 95] and remains among the most useful. The ammonia is best added to the solvent at the bottom of the tank, since this gives the more constant conditions. With this system all the purines and pyrimidines found in nucleic acids may be resolved from each other. However, this system has a low capacity for guanine, and for mixtures containing relatively high concentrations of guanine, acid systems are preferred. Either t-butanol[6] or isopropanol[28] may be used together with hydrochloric acid.

A list of solvents which may be used to effect most separations is given below[94]; in some cases the composition is not exactly that given in the reference but has been modified by Markham[94].

1. *n*-Butanol (860 ml.), water (140 ml.)[4, 82].
2. As solvent 1, but with an ammonia atmosphere (5 per cent by volume ammonia solution, sp.gr. 0·880) added to the liquid in the bottom of the tank.
3. *n*-Butanol (770 ml.), water (130 ml.), 98–100 per cent formic acid (100 ml.)[82].
4. t-Butanol (700 ml. at 26°C), constant boiling hydrochloric acid (132 ml.), water to 1 l. at 20°C[6, 12, 84, 96–98].
5. Isopropanol (680 ml.), concentrated hydrochloric acid (164 ml., sp.gr. 1·19), water to 1 l.[28].
6. Isopropanol (700 ml.), water (30 ml.) with 0·35 ml. of ammonia solution (sp.gr. 0·880) per litre of tank volume[99].
7. Saturated ammonium sulphate in water (800 ml.), 1M sodium acetate (200 ml.), isopropanol (20 ml.)[12].

The first three solvents are suitable for purines, pyrimidines and nucleosides; solvent 2, however, is not suitable for quantitative work with guanine which is best analysed using solvents 3 or 5. Solvent 3 is particularly useful for nucleosides. In all the first three solvents the nucleotides move very little and remain near the origin. Solvent 4 is suitable for studying acid hydrolysates and will separate guanine, adenine, cytidylic acid and uridylic acids. Solvent 5 has been used extensively for the acid and alkaline hydrolysates of nucleic acids, all the bases may be resolved and it is to be particularly recommended for mixtures containing high concentrations of guanine. It also resolves nucleosides, mononucleotides and small oligonucleotides[28, 100–110]. A similar acid solvent, but using *n*-butanol (*n*-butanol, 0·5N hydrochloric acid, 2-methoxyethanol in volume ratio 30 : 17 : 10) has been used by Laland, Overend and Webb[17, 29]. Solvent 6 separates small oligonucleotides and mononucleotides, both in the -2′- or -3′- form and has also been used for the separation of deoxypentose dinucleotides and dinucleoside monophosphates[99, 104]. It has also been employed by Brown and Todd and co-workers for the separation of the cyclic mononucleotides[111] and of nucleotide esters[112]. Solvent 7 is mainly important for the separation of the nucleoside-3′-phosphates from the -2′-phosphates which can be accomplished so long as the purine or pyrimidine possesses an amino group (*i.e.* uridylic acid isomers cannot be resolved). It also separates the cyclic -2′,3′-phosphates from the -2′- or -3′-phosphates[12].

For mixtures that are too complex to resolve by one-dimensional chromatography, a useful two-dimensional system consists of the isopropanol–hydrochloric acid solvent used in combination with the *n*-butanol–ammonia or isopropanol–ammonia solvents.

The R_F values $\left(R_F = \dfrac{\text{movement of band}}{\text{movement of liquid front}}\right)$ for the purines, pyrimidines and nucleosides are given in *Table 4* for solvents 1, 2, 3 and 5[93, 94]. A certain amount of variation in the values must be expected with changes in temperature. In *Table 5* are given the R_F values of the nucleotides in solvents 4, 5 and 7. Also included are the values for a sodium phosphate–isoamyl alcohol system (no. 8) which was used by Carter[80] to resolve the isomeric nucleotides ; the solvent consists of 5 per cent disodium hydrogen phosphate saturated with isoamyl alcohol. Better resolution of the adenosine phosphates in this system has been obtained by substituting the isoamyl alcohol by 0·5 per cent laurylamine in *n*-amyl alcohol[113].

The Separation of Hydrolysis Products by Electrophoresis

The number and variety of ionizable groups in nucleic acids and their components have already been referred to in connection with their adsorption on to ion-exchange columns and on to paper. The difference in the net charge carried by the components at a given pH will cause the components to move at different speeds in an electric field and thus permit a separation of the components to be made. An important feature of the method is that the relative mobilities of the components at a given pH may be predicted quite accurately, thus enabling the components to be identified.

The first attempt to separate by electrophoresis the nucleic acid components produced by enzymic hydrolysis was made by Gordon and Reichard[114] who used agar gel as the supporting medium.

Table 4. R_F values of purines, pyrimidines and nucleosides

	Solvent			
	1	2	3	5
Adenine	0·38	0·28	0·33	0·32
Guanine	0·15	0·11	0·13	0·22
Hypoxanthine	0·26	0·12	0·30	0·29
Xanthine	0·18	0·05	0·24	0·21
Uracil	0·31	0·19	0·39	0·66
Thymine	0·52	0·35	0·56	0·76
Cytosine	0·22	0·24	0·26	0·44
5-Methylcytosine	0·29	0·27	—	0·52
5-Hydroxymethylcytosine	0·13	0·12	—	0·44
Adenosine	0·20	0·22	0·12	0·34
Guanosine	0·15	0·03	0·17	0·30
Inosine	—	0·03	—	0·30
Uridine	0·17	0·08	0·25	0·64
Cytidine	0·12	0·11	0·18	0·45
Adenine deoxypentose nucleoside	0·35	—	—	—
Guanine deoxypentose nucleoside	0·21	—	—	—
Hypoxanthine deoxypentose nucleoside	0·23	—	—	—
Uracil deoxypentose nucleoside	0·38	—	—	—
Thymine deoxypentose nucleoside	0·51	0·40	—	0·81
Cytosine deoxypentose nucleoside	0·23	—	—	0·60
5-Methylcytosine deoxypentose nucleoside	0·25	—	—	—

(G. R. Wyatt: *The Nucleic Acids* Vol. 1 (Ed. E. Chargaff and J. N. Davidson): Academic Press, New York, 1955, p. 252; R. Markham, *Brit. med. Bull.* 1954, **10,** p. 216)

Table 5. R_F values of nucleotides

	Solvent			
	4	5	7	8
Adenosine-2′-phosphate	0·50	0·48	0·26	0·74
Adenosine-3′-phosphate			0·16	0·67
Guanosine-2′-phosphate	0·46	0·43	0·50	0·79
Guanosine-3′-phosphate			0·40	
Uridylic acid	0·80	0·77	0·73	0·85
Cytidylic acid	0·56	0·58	0·73	0·85
Adenosine-5′-phosphate	—	0·43	—	0·69
Deoxycytidylic acid	—	0·64	—	—
Thymidylic acid	—	0·81	—	—

(From G. R. Wyatt: *The Nucleic Acids* Vol. 1 (Ed. E. Chargaff and J. N. Davidson): Academic Press, New York, 1955, p. 256)

This is not a very suitable substance to use and nearly all subsequent work has been carried out on paper. The method has not been used so extensively as that of paper chromatography and its main application has been to the separation of nucleotides, small oligonucleotides and the isomeric nucleotides. It probably provides one of the best methods for separating the four bases from pentose nucleic acid in a single run[93].

Standard procedures for paper electrophoresis may be employed for the hydrolysates of nucleic acids, but convenient techniques, which have been used specifically for nucleic acid components, have been described by Markham and Smith[99, 115] and by Crestfield and Allen[116].

The dissociating groups which are of importance to electrophoretic separation are the primary and secondary phosphoric acid dissociations and the amino groups of guanine, adenine and cytosine. Separations are rarely made in solutions so alkaline that the —NH—CO—, and the sugar hydroxyl dissociations are involved. The basis of the separation of adenylic, guanylic, cytidylic and uridylic acids is the differences in the pK_a' values of the amino groups (see *Table 9*, Chapter 7) which lie between 2 and 5. In this pH range, the primary phosphoric acid groups are fully dissociated and the secondary groups almost totally undissociated. A suitable pH for separation is 3·5 where the degree of dissociation of the amino groups in adenylic acid is 0·54, guanylic acid 0·05, cytidylic acid 0·84 (*Figure 16*) while uridylic acid possesses no amino group. Since each nucleotide carries a unit negative charge due to the fully dissociated phosphoric acid group, the net negative charges are: adenylic acid, 0·46; guanylic acid, 0·95; cytidylic acid, 0·16 and uridylic acid, 1·00. The resistance of the nucleotides to motion through the liquid will be a function of their size and shape, and since these will be approximately the same the above figures should give the relative mobilities of the nucleotides at this pH. With the aid of the dissociation curves (*Figure 16*) the relative mobilities may be calculated at any other pH. The mobility of a nucleotide determines the distance that it will move along the paper between the electrodes during electrophoresis; a mixture of nucleotides at pH 3·5 can thus readily be resolved into four clearly defined spots[115, 117–120].

In *Figure 21*, A, is shown the resolution of the ribonucleotide-3′-phosphates which is obtained in 1 hour with a field of 30 V/cm at pH 3·5 in formate buffer of 0·1 ionic strength. Crestfield and Allen[120] have also shown that resolution can be made at pH 9·2 utilizing the —NH—CO— dissociations of uridylic and guanylic acids

(*Figure 21*, B). This resolution is attributed to the lower pK$'_a$ value of the enolic group of uridylic acid and to the difference in masses between the nucleotides.

Resolution of the isomeric nucleoside-2′-, -3′- and -5′-phosphates was only partially obtained by Davidson and co-workers[117, 121], but Crestfield and Allen[120] have been able to isolate each -5′-phosphate from the corresponding -2′- and -3′-phosphates and also to separate the -2′- and -3′-phosphates. The -5′-phosphates possess two adjacent hydroxyl groups in the ribose moiety whereas the -2′- and -3′-phosphates do not. Complexes between the tetraborate ion and sugars possessing adjacent hydroxyl groups are well known[122], the reaction generally occurring in sodium tetraborate solution at pH 9·2. The complex formed is a stronger acid than boric acid, and hence possesses a high net negative charge at pH 9·2[123]. The

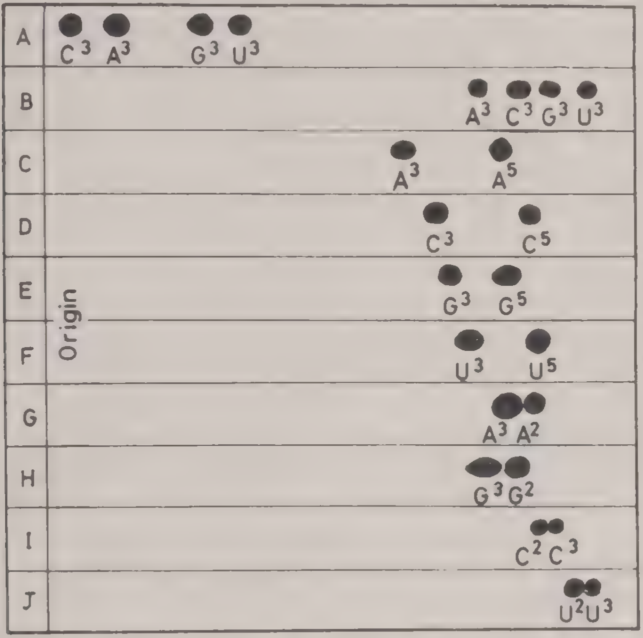

Figure 21. Separation of isomeric ribonucleotides by paper electrophoresis (A, G, C and U refer to adenylic, guanylic, cytidylic and uridylic acids)

A. formate buffer pH 3·5
B–F. 0·1M sodium tetraborate solution pH 9·2
G. 0·1M sodium bicarbonate pH 8
H. formate buffer pH 3·8
I. phosphate buffer pH 6·2
J. phosphate buffer pH 5·8

(A. M. Crestfield and F. W. Allen: *Analyt. Chem.* 1955, **27**, p. 424) mobility of the -5′-phosphate in tetraborate buffer is thus greater than that of the corresponding -2′- and -3′-phosphates. This is found to be the case, as is shown in *Figure 21*, C to F[120, 123]. The dissociation constants of the amino groups of the -2′- and -3′-phosphates are very close but significant differences occur in the values of the secondary phosphoric acid dissociations (*Table 9*, Chapter 7). Resolution of these isomers is most likely to occur at pH values where the dissociations are half complete[124] and Crestfield and Allen[120] have satisfactorily resolved the isomers of the pyrimidine nucleotides in the region of pH 6 (*Figure 21*, I and J). The isomers of the purine nucleotides, however, do not resolve at pH 6 although resolution is to be expected from the calculated net charges on the isomers. This behaviour is attributed by Crestfield and Allen[120] to a steric difference between the two isomers which counteracts the charge difference. Resolution could, however, be effected at pH 8 (*Figure 21*, G). The isomers of guanylic acid separate at neither pH 6, nor pH 8, but separation can be effected at pH 3·8 in formate buffer[120].

Since the separation of the nucleotides has been effected primarily through the differences in the amino dissociation, similar resolutions should be possible with the nucleosides, purines and pyrimidines because, although the dissociation constants are not identical with those of the corresponding nucleotides, the differences between the amino dissociations persist[125]. However, few attempts to separate these substances have been made, presumably because they can be more readily separated by chromatography. At pH 3·5 the bases and nucleosides would all be positively charged, except uridine and uracil which possess no amino group and would be expected to migrate to the cathode in the reverse order to that in which the nucleotides move towards the anode. However, the only electrophoretic study of these substances shows that the migration of the bases at pH 3·2 is not that expected[126]. The nucleosides may also be resolved satisfactorily by complexing with tetraborate at pH 9·2[127].

Partial breakdown of nucleic acids to nucleotides and polynucleotides of low molecular weight may be effected by both chemical and enzymic hydrolysis. The direct separation of such a mixture, which may contain as many as thirty different substances, by electrophoresis alone would be impossible, but by first carrying out a fractionation by paper chromatography and then resolving the individual fractions by electrophoresis, a complete separation of the complex mixture may be effected. The electrophoretic mobility of polynucleotides has been studied by Markham and

Smith[104, 115, 125], who have introduced a method for calculating the relative mobilities (generally relative to uridylic acid as 1·00) of small polynucleotides, which greatly facilitates the recognition of these substances in complex mixtures. A dinucleotide with twice the charge and twice the size of a nucleotide does not have the same electrophoretic mobility as a nucleotide, since the resistance to the motion of a molecule through a fluid is not a direct function of its size. Nucleotides are asymmetric molecules, and from diffusion data[114] it would appear that they possess an axial ratio of about 5 : 1. Consequently a nucleotide will have an axial ratio of between 2·5 : 1 and 10 : 1. Using these limits and assuming that the resistance to motion is the same as in translational diffusion the ratio of the frictional resistance of the dimer to that of the monomer is found to be between 1·32 and 1·85. The observed relative mobilities on paper of the mono-, di- and trinucleotides of uridylic acid, after correction for endosmotic flow are 1 : 1·4 : 1·5 at pH 3·5[104]. At this pH the net charges of the nucleotides are 1, 2 and 3 respectively so that if the frictional resistance to motion of a mononucleotide is taken as 1, then that of the dinucleotide will be 2/1·4 = 1·43, and that of the trimer 3/1·5 = 2. From these values and the values of the net charge obtained from dissociation curves, the mobilities of any mono-, di- or trinucleotide may be calculated for any pH value. For example, where A, G, C and U represent adenylic, guanylic, cytidylic and uridylic acid residues respectively, mobilities at pH 3·5 are as follows:

AG = (0·46 + 0·95)/1·43 = 0·99 times that of uridylic acid
AC = (0·46 + 0·16)/1·43 = 0·43 times that of uridylic acid
AAU = (0·46 + 0·46 + 1·0)/2 = 0·96 times that of uridylic acid

In making these calculations, it is assumed that the different mononucleotides are of the same size and shape and that the dissociation constants are the same in the di- and trinucleotides as in the mononucleotides. The validity of the method has been demonstrated by Markham and Smith[104] who observed for nineteen mono- and polynucleotides a good linear relationship between the observed and the calculated relative mobility. The method has been extensively used by Markham and Smith to study the enzymic hydrolysis products of pentose[99, 104, 115] and deoxypentose[107, 108] nucleic acids.

It is evident that the values of the relative mobilities of the mono-, di- and trinucleotides of a single nucleotide species form a converging series, so that separation of tetra- and higher polynucleotides is not possible. The cyclic dinucleotides, however, which are formed in very small amounts during weak alkaline hydrolysis of pentose nucleic acid[99] and during ribonuclease action[99, 105, 115], may be isolated in this manner.

The Chemical Fractionation of Purines and of Pyrimidine Nucleotides

The hydrolysis of pentose nucleic acids with 1N acid at 100°C for 1 hour as previously described, yields a mixture of purines and pyrimidine nucleotides. The purines form highly insoluble silver salts in acid solution whereas the pyrimidine nucleotides do not and it is thus possible to separate the hydrolysis products[13]. If the purines are redissolved in dilute hydrochloric acid and the pyrimidine nucleotide fraction, after the removal of the silver ions, is treated with prostatic phosphatase, two mixtures, one containing adenine and guanine and the other cytidine and uridine, are obtained. The ultra-violet absorption spectra of the individual members of each pair are sufficiently distinct to permit the concentration of each component to be determined spectrophotometrically[13, 128]. This fractionation, while yielding an analytical method for bases of wide application, does not lend itself to the isolation of the components.

THE COMPOSITION OF NUCLEIC ACIDS

The proportions of the constituent nucleotides in a sample of nucleic acid may be determined by one or other of the methods just described. The method which has been most frequently used is the chromatography on paper of the purine and pyrimidine bases, obtained by the appropriate hydrolytic procedure, although the other procedures have also been employed. These methods of analysis have been extensively applied to samples of nucleic acids from a large number of different tissues and the results have been tabulated by Chargaff[129] and by Magasanik[130]. The evaluation of the very considerable amount of data, based on the work of many different groups of workers, using different preparations, not infrequently prepared by different methods, is difficult. The results, however, have served a very considerable purpose in the development of our knowledge of the nucleic acids, in that the existence of differences in the composition of nucleic acids from different sources has been established beyond doubt.

However, the differences that have been observed in the composition of the various fractions of deoxyribose nucleic acid (Chapter 3, *Table 1*, p. 53) indicate that the over-all composition of a nucleic acid may not have a great deal of significance unless it is quite certain that no fractionation occurred during the isolation procedure. Even when it is certain that the nucleic acid sample is representative of the total nucleic acid of the cell, it is evident that its composition will be of much less significance than the composition of the single fractions each of which may represent a single biological unit in the cell. However, the variations in the composition of the various fractions shown in *Table 1* are not very great compared with the variations in the composition of deoxypentose nucleic acids from various sources. The latter variations do not therefore, as yet, invalidate the conclusion that the total analysis of different cells is characteristic of that cell.

Representative values of the analysis of deoxypentose nucleic acids from different tissues are given in *Table 6*. As Chargaff[129] has

Table 6. The composition of some deoxypentose nucleic acids; molar proportions per 100 g-atoms of phosphorus in hydrolysate, corrected for 100 per cent recovery

Tissue	*Adenine*	*Guanine*	*Cytosine*	*5-Methyl cytosine*	*Thymine*	$\frac{\text{AD}}{\text{THY}}$	$\frac{\text{GU}}{\text{CY}+\text{MCY}}$	$\frac{\text{AD}+\text{THY}}{\text{GU}+\text{CY}+\text{MCY}}$	*Actual recovery* %
Calf thymus[131]	27·3	22·7	21·6	—	28·4	0·96	1·05	1·26	93·7
Calf thymus[25]	28·0	23·5	20·4	—	28·1	1·00	1·15	1·28	100·2
Calf thymus[16]	29·2	20·8	20·8	—	29·2	1·00	1·00	1·40	96·0
Calf thymus[28]	28·2	21·5	21·2	1·3	27·8	1·02	1·01	1·27	90·0
Calf thymus[29]	28·0	20·9	21·4	1·9	27·8	1·01	0·96	1·26	91·0
Calf thymus[67]	27·4	21·4	21·0	1·1	29·1	0·94	0·97	1·30	99·6
Beef spleen[30]	27·9	22·7	20·8	1·3	27·3	1·02	1·02	1·23	100·0
Beef spleen[67]	27·7	22·1	21·8	—	28·4	0·97	1·01	1·28	98·0
Beef liver[132]	28·8	21·0	21·1	—	29·0	0·99	1·00	1·37	94·1
Beef pancreas[67]	27·8	21·9	21·7	—	28·5	0·97	1·01	1·29	97·8
Beef kidney[25]	28·3	22·6	20·9	—	28·2	1·00	1·08	1·30	96·9
Beef sperm[28]	28·7	22·2	20·7	1·3	27·2	1·05	1·01	1·26	90·0
Sheep thymus[132]	29·3	21·4	21·0	—	28·3	1·03	1·02	1·36	94·3
Sheep liver[132]	29·3	20·7	20·8	—	29·2	1·00	1·00	1·41	89·9
Sheep spleen[25]	28·0	22·3	21·1	—	28·6	0·98	1·06	1·30	92·9
Sheep sperm[28]	28·8	22·0	21·0	—	27·2	1·06	1·05	1·27	88·5
Man thymus[132]	30·9	19·9	19·8	—	29·4	1·05	1·00	1·52	94·6
Man liver[132]	30·3	19·5	19·9	—	30·3	1·00	0·98	1·53	95·4
Man spleen[67]	29·2	21·0	20·4	—	29·4	0·99	1·03	1·42	96·6
Man sperm[133]	30·9	19·1	18·4	—	31·6	0·98	1·04	1·67	96·0
Herring sperm[28]	27·8	22·2	20·7	1·9	27·5	1·01	0·98	1·23	91·0
Salmon sperm[134]	29·7	20·8	20·4	—	29·1	1·02	1·02	1·43	94·2
Abacia lixula sperm[135]	31·2	19·1	19·2	—	30·5	1·02	1·00	1·61	94·2
Echinocardium cordatum sperm[135]	32·9	17·0	17·9	—	32·2	1·02	0·95	1·86	96·4
Sarcina lutea[17]	13·4	37·1	37·1	—	12·4	1·08	1·00	1·02	—
Wheat germ[136]	27·3	22·7	16·8	6·0	27·1	1·01	0·99	1·19	96·2
Yeast[69]	31·3	18·7	17·1	—	32·9	0·95	1·09	1·79	92·0
Pneumococcus type III[25]	29·8	20·5	18·0	—	31·6	0·94	1·14	1·59	92·5
Vaccinia virus[30]	29·5	20·6	20·0	—	29·9	0·98	1·03	1·46	100·0
$T2r^+$ phage[30]	32·0	18·0	—	16·8*	33·3	0·96	1·07	1·88	97·0

* 5-Hydroxymethylcytosine

pointed out, there appear to be three major types of acid, the first in which adenine and thymine predominate, the second in which guanine and cytosine are the major constituents, and the third where all four constituents are present in roughly the same proportion. The observation that in a very large number of cases the ratios of adenine to thymine and of guanine to cytosine are very close to unity, has been used to support the helical structure proposed by Watson and Crick (see Chapter 9) in which specific hydrogen bonds between these pairs of bases are postulated. If every adenine residue is so bonded to thymine and every guanine to cytosine, the ratios would be unity. However, in view of the findings of Bendich and his co-workers given in *Table 1* (p. 53) which show that these ratios are significantly different from unity in some of the fractions obtained from deoxyribose nucleic acid of calf thymus, it would appear that the value of unity obtained for these ratios from the total analysis of nucleic acid may have been fortuitous and this evidence does not necessarily support the Watson–Crick model.

In *Table 7* are given some characteristic values for the analysis of various pentose nucleic acids. The ratio of purines to pyrimidines varies considerably and only in relatively few nucleic acids does it appear to be close to unity. Other ratios of the bases have not been given in *Table 7* as it is doubtful whether they would have any significance in view of the fact that few of the results refer to the total pentose nucleic acid of the cell. Elson and Chargaff[138, 142–144] have developed techniques for the isolation of the total pentose nucleic acid from viruses and tissue and some of their results are given in *Table 23* (Chapter 12, p. 286) where it is seen that the ratios of adenine to uracil and of guanine to cytosine, while not unity, are approximately equal in each sample. The possible significance of this result is discussed in Chapter 12.

The analytical procedures described have not only enabled accurate analyses of the various nucleic acids to be made but also have led to the discovery of the presence of new nucleotides, generally present only in small amounts in the nucleic acids. The first of these to be discovered was 5-methylcytosine which was first isolated as the picrate from nucleic acid hydrolysates by Johnson and Coghill[146] using classical methods. Its presence in several deoxypentose nucleic acids has now been confirmed by chromatographic methods[4, 28, 67, 136] (see *Table 6*). 5-Hydroxymethylcytosine is present in phage deoxypentose nucleic acids in quite large amounts[30]. More recently a new, as yet unidentified, nucleotide has been discovered in the hydrolysate of yeast ribonucleic acid, and N-methylated purines and 1-methylguanine have been identified in the same nucleic acid[147, 148].

Table 7. The composition of some pentose nucleic acids; molar proportions per 100 g-atoms of phosphorus in hydrolysate, corrected for 100 per cent recovery

Tissue	*Adenine*	*Guanine*	*Cytosine*	*Uracil*	$\frac{AD+GU}{CY+UR}$
Bakers' yeast[18]	25·1	30·1	20·1	24·7	1·23
Bakers' yeast[7]	25·7	28·1	21·8	24·4	1·16
Bakers' yeast[137]	25·0	28·0	20·0	27·0	1·13
Escherichia coli[138]	27·0	27·5	23·0	22·5	1·20
Rabbit liver[121]	19·3	32·6	28·2	19·9	1·08
Rat liver[121]	19·1	33·5	26·6	20·8	1·10
Rat liver[139]	19·0	34·0	30·2	16·8	1·13
Sheep liver[18]	20·4	39·4	25·8	14·4	1·49
Man liver[18]	11·4	44·4	31·6	12·6	1·26
Calf liver[18]	21·6	40·6	23·3	14·5	1·65
Calf liver[140]	19·5	35·0	29·1	16·4	1·20
Calf pancreas[140]	14·2	48·6	23·8	13·4	1·69
Calf spleen[140]	17·9	35·2	31·6	15·3	1·13
Calf thymus[140]	18·5	43·9	25·6	12·0	1·65
Tobacco mosaic virus (TMV)[91]	29·8	25·4	18·5	26·3	1·24
Cucumber (CV3)[91]	25·7	25·5	18·2	30·6	1·05
Tomato bushy stunt (BS)[141]	27·6	27·6	20·4	24·4	1·23
Turnip yellow mosaic (TY)[13]	22·6	17·2	38·0	22·2	0·66
Southern bean mosaic (SBM)[141]	25·9	25·9	23·0	25·2	1·07
Potato X (PX)[141]	34·4	21·4	22·8	21·4	1·27
Sarcina lutea[17]	16·7	28·4	32·9	22·0	0·82

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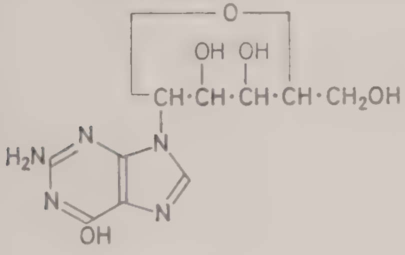
5

THE STRUCTURE AND SYNTHESIS OF THE NUCLEOSIDES

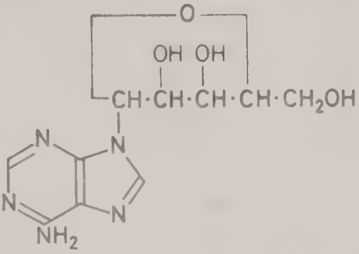
INTRODUCTION

THE repeating units in the nucleic acids, equivalent in many respects to the amino acids in the proteins, are the nucleotides, which in addition to existing in the polymerized form in nucleic acids, may also be present in cells in the monomolecular state as vital co-enzymes. The nucleotides are the phosphoric esters of the nucleosides which are purine or pyrimidine glycosides and which do not generally exist in cells in the free state. The nucleosides and nucleotides may be prepared either by the chemical or enzymic hydrolysis of nucleic acids, as described in Chapter 4, or, in many cases, by synthesis.

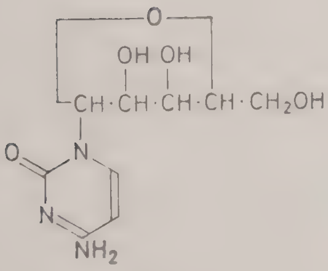
Nearly all the structural features of the nucleosides have now been determined. The nucleosides present in ribonucleic acids are



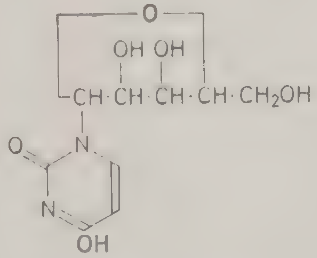
(I) Guanosine



(II) Adenosine



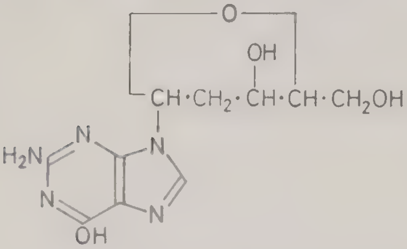
(III) Cytidine



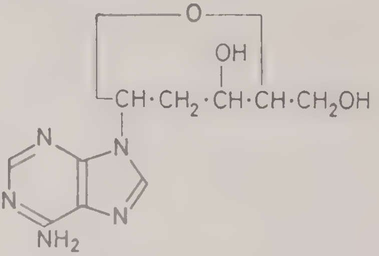
(IV) Uridine

guanosine (*I*), adenosine (*II*), cytidine (*III*) and uridine (*IV*). Guanosine and adenosine, which were the first two nucleosides to be isolated from nucleic acids, are the 9-β-D-ribofuranosides of guanine and of adenine respectively. Cytidine and uridine are the 3-β-D-ribofuranosides of cytosine and uracil.

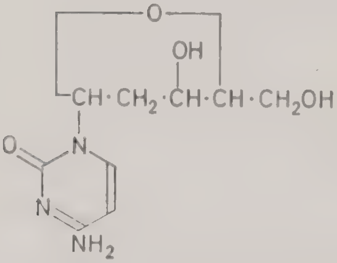
The purine nucleosides present in deoxyribose nucleic acid are deoxyguanosine (guanine deoxyriboside) (*V*) and deoxyadenosine (adenine deoxyriboside) (*VI*). The configuration of the glycosidic linkage has only been determined for deoxyadenosine which was found to be a β-glycoside. Although a similar study has not been made on deoxyguanosine, it appears virtually certain that since deoxyadenosine and deoxycytidine and thymidine (see below) are all β-glycosides, that all the natural deoxyribonucleosides have this configuration[1]. Deoxyguanosine and deoxyadenosine are thus 9-β-D-(2′-deoxy)ribofuranosides of guanine and adenine respectively. The main pyrimidine nucleoside components of deoxyribose nucleic acid are deoxycytosine (cytosine deoxyriboside) (*VII*) and thymidine (thymine deoxyriboside) (*VIII*). These are the 3-β-D-(2′-deoxy)ribofuranosides of cytosine and thymine respectively. Several deoxypentose nucleic acids contain small amounts of 5-methylcytosine deoxyriboside (*IX*) and uracil deoxyriboside (*X*)



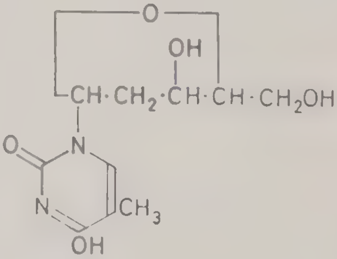
(V) Deoxyguanosine



(VI) Deoxyadenosine

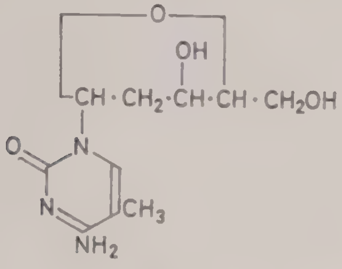


(VII) Deoxycytosine

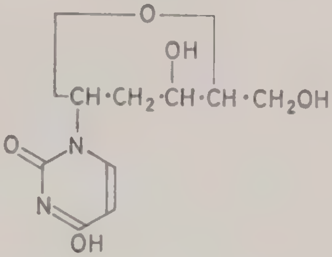


(VIII) Thymidine

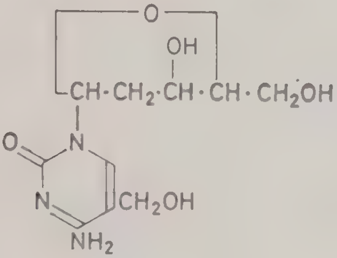
(this may be an artifact arising out of the bacterial deamination of deoxycytosine[2]) and the deoxypentose nucleic acids of bacteriophage contain 5-hydroxymethylcytosine deoxyriboside (*XI*) which completely replaces deoxycytosine in these acids. Although no definite structural studies have been made, these three nucleosides are all most probably the 3-β-D-(2′-deoxy)ribofuranosides of the corresponding bases.



(IX)
5-Methylcytosine deoxyriboside



(X)
Uracil deoxyriboside



(XI)
5-Hydroxymethylcytosine deoxyriboside

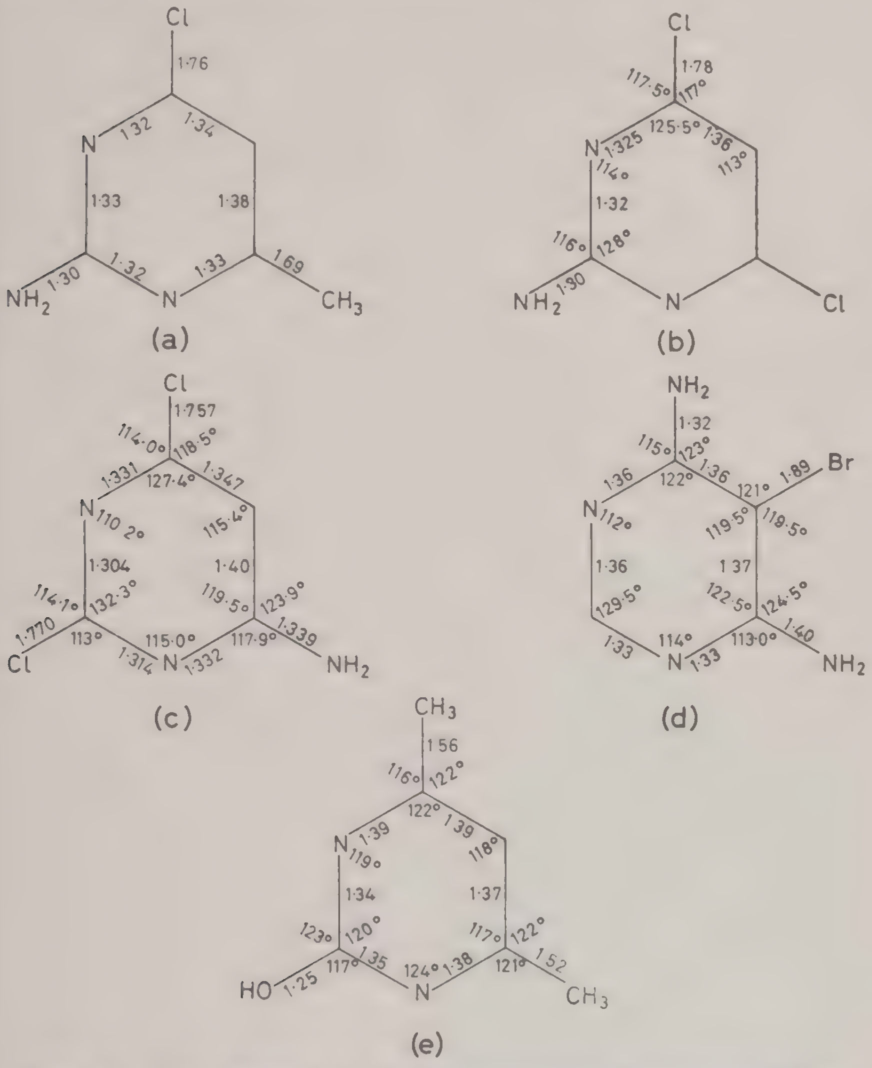
THE NATURE AND STRUCTURE OF THE PURINES AND PYRIMIDINES

The purines and pyrimidines were first isolated from the hydrolysates of the nucleosides and identified by the classical methods of organic chemistry. As we have seen in Chapter 4, these operations are now much more readily carried out by the methods of chromatography. Guanosine was, historically, the first of the nucleosides to be isolated, being discovered by Schulze and Bosshard[3] in 1885. On hydrolysis with dilute acid it was shown to give guanine (2-amino-6-hydroxypurine) by Levene and Jacobs[4]. Adenosine was first isolated from nucleic acid by Levene and Jacobs[5], who showed that it yielded adenine (6-aminopurine) on acid hydrolysis. Adenine had previously been isolated from the acid hydrolysate of the nucleic acid of beef pancreas by Kossel[6,7] and its structure determined by partial synthesis from 2,6,8-trichloropurine by Fischer[8]. On strong acid hydrolysis, uridine and cytidine were found to yield uracil (2,4-dihydroxypyrimidine) and cytosine (4-amino-2-hydroxypyrimidine) respectively[9]. Adenine, guanine, cytosine and thymine were first obtained from the respective deoxyribonucleosides on acid hydrolysis by Levene and London[10] and Klein[11].

Until 1925, adenine, guanine, cytidine, uracil and thymine were the only known bases in nucleic acids. In that year, Johnson and Coghill[12] described the occurrence of 5-methylcytosine, which they isolated as the picrate, in the hydrolysis products of a nucleic acid from tubercle bacilli. Its presence as a minor pyrimidine constituent (about 1–3 per cent of the total base composition, see *Table 6*, Chapter 4) of the deoxypentose nucleic acids from several sources has now been confirmed by chromatographic methods[13–16]. In the deoxypentose nucleic acid of wheat germ it is present in a rather higher proportion (6 per cent of total bases)[16]. Following the observation of the absence of cytosine in the deoxypentose nucleic acid of *Escherichia coli* phage T2[17], Wyatt and Cohen[18,19] discovered that 5-hydroxymethylcytosine completely replaces cytosine in the deoxypentose nucleic acids of the T-even phages.

The detailed structures of the pyrimidines and purines are not only of importance in determining the structures of the nucleosides and nucleotides, but are of equal importance in determining the structure of the nucleic acids. To give an example: whether the —NH—CO— group exists in the keto or enolic form is important to the formation of the specific hydrogen bonds between base pairs, which is an important feature of the Watson–Crick model for deoxypentose nucleic acid; this structure also hinges on the configuration of the glycosidic linkage being identical in all the nucleotides.

The full details of the structures of the five pyrimidines known to occur in the nucleic acids have not been determined by x-ray methods although preliminary results on the unit cell dimensions of thymine have been recorded[20]. The structures of some related pyrimidine derivatives, 2-amino-4-methyl-6-chloropyrimidine, 2-amino-4,6-dichloropyrimidine, 4-amino-2,6-dichloropyrimidine, and 5-bromo-4,6-diaminopyrimidine have been determined by Clews and Cochran[21,22] and details are shown in *Figure 22*. The pyrimidine ring is considered to be planar[21–23] and the C—N and C—C bond distances correspond to approximately 50 per cent double bond character, as would be expected for a six-membered ring in which resonance of the benzene type is possible. Whether such resonance can occur in the amino- and hydroxypyrimidines found in the nucleic acids depends on whether the amino- and hydroxy- groups exist in the amino or imino and the enol or keto forms, respectively. An amino group in position 6 or 4 of the pyrimidine nucleus may show reduced amino behaviour if a


Cl
1·76
1·32
1·34
N
1·33
1·38
1·30
1·32
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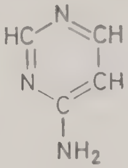
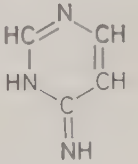
169
NH_2
N
CH_3
(a)
Cl
1·78
117·5°
117°
125·5°
1·36
N
1·325
114°
113°
1·32
116°
128°
1·30
NH_2
N
Cl
(b)
Cl
1·757
114·0°
118·5°
1·331
127·4°
1·347
N
110·2°
115·4°
1·304
1·40
114·1°
132·3°
119·5°
123·9°
1·770
113°
115·0°
117·9°
1·339
Cl
1·314
N
1·332
NH_2
(c)
NH_2
1·32
115°
123°
1·36
122°
1·36
121°
1·89
Br
N
112°
119·5°
119·5°
1·36
1·37
129·5°
122·5°
124·5°
1·33
114°
113·0°
1·40
N
1·33
NH_2
(d)
CH_3
1·56
116°
122°
122°
1·39
1·39
N
119°
118°
1·34
1·37
123°
120°
117°
122°
1·35
117°
124°
1·38
121°
1·52
HO
1·25
N
CH_3
(e)

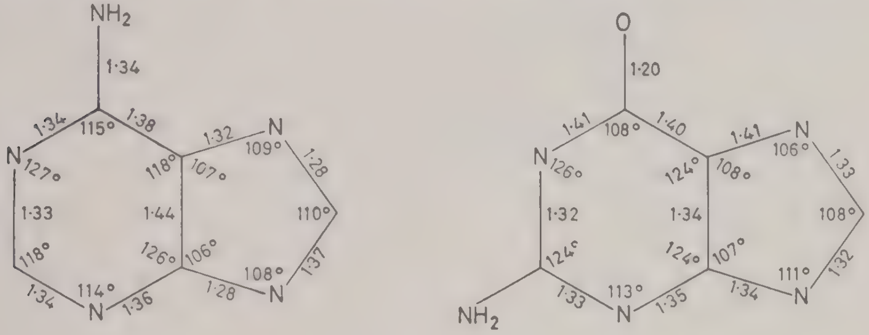
Figure 22. The structure of (*a*) 2-amino-4-methyl-6-chloropyrimidine, (*b*) 2-amino-4,6-dichloropyrimidine, (*c*) 4-amino-2,6-dichloropyrimidine, (*d*) 5-bromo-4,6-diaminopyrimidine, (*e*) 2-hydroxy-4,6-dimethylpyrimidine ((*a–d*) C. J. B. Clews and W. Cochran: *Acta Cryst.* 1948, **1**, p. 10; *Acta Cryst.* 1949, **2**, p. 55. (*e*) From G. J. Pitt: *Acta Cryst.* 1948, **1**, p. 172)

tautomeric change occurs converting the 6-aminopyrimidine (*XII*) into the corresponding iminodihydropyrimidine (*XIII*). The x-ray evidence[22] shows that in the crystalline state, 4-amino-2,6-dichloropyrimidine and 5-bromo-4,6-diaminopyrimidine are in the amino form. It is therefore probable that cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine are also in the amino form. Information concerning the position of the hydrogen atom in the —NH—CO— groups, *i.e.* whether they exist in the keto or enol form may be obtained from the length of the C—O bond in 2-hydroxy-4,6-dimethylpyrimidine determined by Pitt[24]. The structural details of this compound are also shown in *Figure 22* (*e*). The length of the C—O bond is 1·25 ± 0·04 Å which is quite different from the corresponding bond length of the phenolic hydroxyl group in resorcinol (1·36–1·37 Å) but is much closer to the C—O bond distance in oxalic acid dihydrate (1·24–1·30 Å). In the crystal therefore, the C—O bond possesses considerable double bond character although the hydrogen atom is still covalently bound to the oxygen atom and takes part in the hydrogen bond between the oxygen atom and a water molecule. In aqueous solution, however, the position of the hydrogen atom is not necessarily the same as in the crystal and evidence from the ultra-violet absorption spectra of solutions of various pyrimidines shows that the keto form predominates in solution. Early work confused enolization of the —NH—CO— group with ionization[25–27], but Marshall and Walker[28], by measuring the ultra-violet absorption spectra of a number of 2- and 4(6)-substituted pyrimidines and the corresponding $N_{(1)}$- and $N_{(3)}$-methylated derivatives in aqueous solutions of pH values so determined that the substances were either all present as neutral molecules or ions, conclude that all potential 2- and 4(6)-hydroxypyrimidines should be represented structurally as being in the keto form, *i.e.* as pyrimidones. Thus uracil should be regarded as 2,4(6)-pyrimidinedione.


(*XII*)


(*XIII*)

The structure of adenine hydrochloride was first studied by Bernal and Crowfoot[29], but no attempt was made to determine the positions of the atoms in the unit cell. More detailed studies, however, have been made by Broomhead[30, 31], and by Cochran[32] for both adenine and guanine hydrochloride. The bond distances and bond angles are given in *Figure 23*. The structures of both molecules are very similar, the main differences being that the amino nitrogen atom ($N_{(10)}$) of guanine is displaced by 0·11 Å from the plane containing the other atoms and that the $C_{(4)}$—$C_{(5)}$ bond distance is longer in adenine by 0·10 Å. This difference, which could be due to experimental error[31], is in agreement with the different acid–base properties of adenine and guanine (see Chapter 7, p. 135). The $C_{(6)}$—O bond has a length (1·20 Å) which suggests that it possesses predominantly double bond character, thus confirming the conventional keto formula ascribed to guanine. However, an error in this value, of only — 0·05 Å (*i.e.* giving a bond length of 1·25 Å) would make the bond of a comparable length to that of the C—O bond in glycine and in diketopiperazine, where the bond possesses only 50 per cent double bond character.



(a) (b)

Figure 23. The structure of (*a*) adenine, (*b*) guanine (J. M. Broomhead: *Acta Cryst.* 1948, **1**, p. 327; *Acta Cryst.* 1951, **4**, p. 97)

The positions of the hydrogen atoms in the adenine and guanine cation in the crystalline state have been considered by both Broomhead[31] and Cochran[32]. In the adenine cation hydrogen atoms are at $N_{(1)}$, $C_{(2)}$, $C_{(8)}$, $N_{(9)}$, and two are at $N_{(10)}$. The positive charge may be located at $N_{(1)}$, $N_{(9)}$ or $N_{(10)}$ and the short length of the $C_{(6)}$—$N_{(10)}$ bond (1·34 Å compared with 1·47 Å for a single bond) may be attributed to the contribution of resonance forms in which the $N_{(10)}$ bears a positive charge and the $C_{(6)}$—$N_{(10)}$ bond is a double bond. The location of the hydrogen atoms in the guanine cation is known less precisely. Four hydrogen atoms are at $N_{(1)}$, $N_{(9)}$, $N_{(10)}$ and $C_{(8)}$ and hydrogen bonds exist in the crystal between $N_{(3)}$ and $N_{(10)}$ and between O and $N_{(7)}$. There are thus four possible tautomeric forms. In aqueous solution the distinction between such tautomers is of little meaning.

THE NATURE AND STRUCTURE OF THE CARBOHYDRATE COMPONENTS

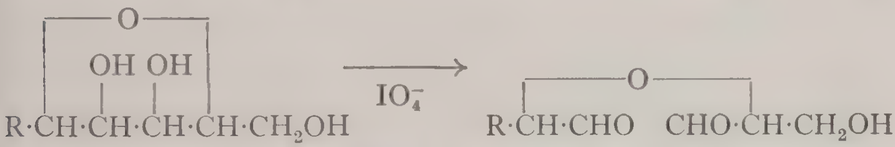
The carbohydrate component of pentose nucleic acid was described by Hammarsten[33], on the basis of purely qualitative tests and the properties of the osazone, as a pentose. Levene and Jacobs[34] succeeded in isolating the sugar in a crystalline form and reported that it was a new sugar resembling arabinose, but differing from it in several important respects. They concluded that this new sugar was D-ribose. Full identification came with the synthesis by van Ekenstein and Blanksma[35] of L-ribose using essentially the same method as Fischer and Piloty[36], who had earlier prepared ribose in an amorphous form. The nature of the sugar in all four of the nucleotides from yeast ribonucleic acid has now been confirmed by Gulland and Barker and co-workers[37–40] by converting the sugar components of the separate nucleotides into the D-ribobenzimidazole.

The sugar component of the deoxypentose nucleic acids proved difficult to identify, both because of the marked lack of stability of the sugar in acid solution and the difficulties in effecting its synthesis. Acid hydrolysis under similar conditions to those used in isolating the sugar from the ribonucleotides caused conversion of the sugar into levulinic acid. However, by using dilute acid for a short period of time it was possible to obtain a deoxypentose in crystalline form[10, 41]. By examination of derivatives and comparison with synthetic L-2-deoxyribose it was concluded that the sugar isolated was D-2-deoxyribose[42].

More recently, Laland and Overend[43] and Jones and Laland[44], have isolated D-2-deoxyribose from the deoxyribose nucleic acids of cod and herring roe and of *Mycobacterium phlei*. The sugar has been characterized by mixed melting points with authentic D-2-deoxyribose, optical rotation and the preparation of the aniline-D-deoxyriboside which had previously been characterized[45, 46]. The sugar component of the deoxyribonucleic acid of calf thymus has also been isolated from the nucleosides by resin hydrolysis, enzymic hydrolysis and amalgam reduction followed by acid hydrolysis and characterized through the benzylphenylhydrazone by Walker and Butler[47]. They also concluded that the sugar is D-2-deoxyribose.

The furanose structure of the sugar residues in both the ribonucleosides and the deoxyribonucleosides has been determined in a number of ways. Acetylation of adenosine or guanosine followed by methylation and deacetylation yields a trimethyl-N-methyl derivative which on hydrolysis with dilute acid gives the appropriate N-methylpurine and trimethylribose[48]. The structure of the methylated ribose was established by oxidation to 2,3,5-trimethyl-γ-D-ribolactone and then to *meso*-dimethoxysuccinic acid and also by comparison with synthetic 2,3,5-trimethyl-D-ribofuranose[49]. A similar method determined the furanose structure of the sugar residue in the pyrimidine nucleosides[50]. The furanose structure has been confirmed by the reaction of the ribonucleosides with trityl chloride studied by Bredereck[51–54] and Levene and Tipson[55, 56]. Furthermore, reaction of adenosine, guanosine, inosine and uridine with acetone in the presence of acidic dehydrating agents yields the appropriate 2′,3′-isopropylidene derivative[56, 57]. Both the derivatives of inosine and uridine form 5′-tosyl derivatives, which on reaction with sodium iodide yield the 5′-iodo compounds. These reactions are in agreement with the furanose structure.

Further evidence for the furanose structure comes from the action of periodate on the nucleosides and deoxyribonucleosides. The oxidation of the ribonucleosides involves the consumption of 1 mole of periodate per mole of nucleoside and converts the ribonucleoside into the corresponding dialdehyde with no simultaneous liberation



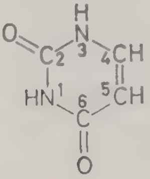
$$R\cdot CH\cdot \underset{}{\overset{OH}{C}}H\cdot \overset{OH}{C}H\cdot CH\cdot CH_2OH \xrightarrow{IO_4^-} R\cdot CH\cdot CHO \quad CHO\cdot CH\cdot CH_2OH$$

of formic acid. This reaction is in agreement with the furanose structure of the sugar[58]. With deoxyribonucleosides no oxidation occurs indicating the absence of the *cis*-1,2-glycol system in these compounds, which is again in agreement with the furanose structure[59].

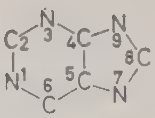
THE POSITION OF THE GLYCOSIDIC LINKAGE

Since cytidine may be deaminated with nitrous acid to give uridine[60] it follows that the position of the glycoside linkage in the two nucleosides is the same, and furthermore it indicates that this linkage does not involve the substituent at position 6 in these nucleosides. Of the remaining four positions which could be occupied in uracil (*XIV*) by the glycoside linkage, position 1 may be rejected as uridine may be converted into N-methyluridine which yields 1-methyluracil on hydrolysis[61]. Position 5 may be excluded since uridine can be converted to 5-nitrouridine and

5-bromouridine which yield the corresponding uracil derivatives on hydrolysis[62], and positions 4 and 5 may also be excluded since



(XIV) Uracil

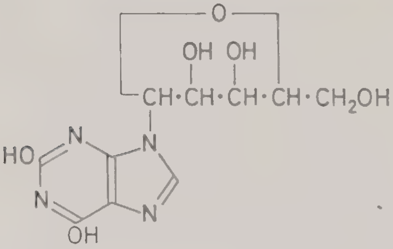


(XV) Purine nucleus

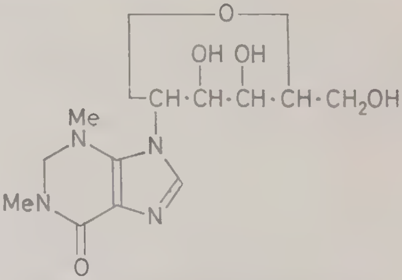
the formation of 4,5-diphenyl hydrazinouridine by the action of bromine followed by phenylhydrazine on uridine indicates the absence of constituents on these positions[63]. The only remaining position is at 3 and thus it appears that uridine and cytidine are 3-glycosides.

The structure of cytidine has also been determined by Furberg[64] by x-ray methods (see p. 110) and the position of the glycosidic linkage at $N_{(3)}$ confirmed. Similar arguments have been employed by Bredereck, Müller and Berger[65] to the case of thymidine which is thus also an $N_{(3)}$ glycoside and the structure has been confirmed by the x-ray study of Huber[66].

With the purine nucleosides, the ease of hydrolysis in acid solution suggested that the linkage between purine and sugar involved a C—N rather than a C—C bond. Since the purine nucleosides can be readily deaminated by nitrous acid without affecting the glycosidic linkage[60, 67–69] it is evident that the amino groups of adenine and guanine are not involved.



(XVI) Xanthosine



(XVII) Theophylline riboside

The possible positions are therefore 1, 3, 7 and 9 (*XV*). Positions 1 and 3 are excluded since methylation of xanthosine (*XVI*), obtained by the deamination of guanosine, gives theophylline riboside (*XVII*)[69]. Of the two remaining positions, 7 and 9, it was first assumed that the glycosidic linkage was at $N_{(7)}$, this, however, was later corrected by Gulland and co-workers[67, 70, 71] to $N_{(9)}$, on the grounds that the absorption spectra of adenosine and inosine and of guanosine and xanthosine much more closely resemble the spectra of the 9- than the 7-methylpurines. Since deoxyadenosine[72] and deoxyguanosine[70] give similar spectra to adenosine and guanosine, it was concluded also that these nucleosides must also be $N_{(9)}$-glycosides. Although the detailed structure of the purine nucleosides has not been determined by x-ray methods a preliminary study by Furberg[73] has confirmed the position of the glycosidic linkage at $N_{(9)}$.

Confirmation of the position of the glycosidic linkage in both the purine and pyrimidine nucleosides and deoxynucleosides has come from the extensive studies of Todd and co-workers on the configuration of the glycosidic linkage and on the synthesis of the nucleosides (see below).

THE CONFIGURATION OF THE GLYCOSIDIC LINKAGE

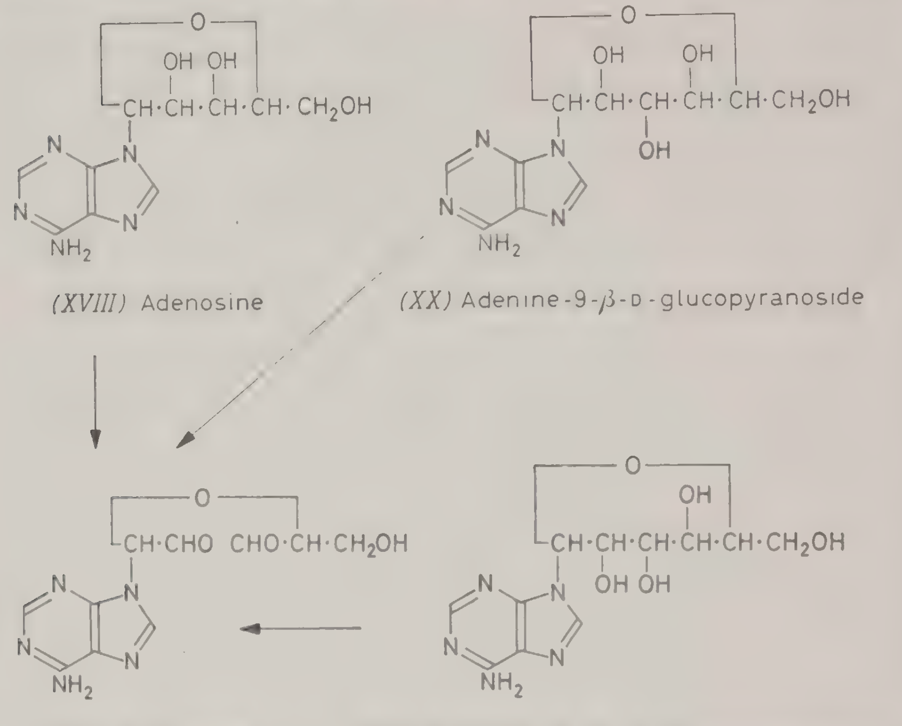
The oxidation of nucleosides by periodate to give the corresponding dialdehyde and formic acid, coupled with the synthesis of new nucleotides of unambiguous structure, has enabled the configuration of the glycosidic linkage to be determined and at the same time has confirmed the location of the sugar residue.

The dialdehydes (α-(adenine-9)-α′-hydroxymethyldiglycollic dialdehyde) (*XIX*) produced by the oxidation by periodate of adenosine (*XVIII*) and adenine-9-β-D-glucopyranoside (*XX*) were found to be identical by Davoll, Lythgoe and Todd[74]. Although the β-configuration of the glucoside could not be fixed with absolute certainty, since the assumption was made that Walden inversion occurs during synthesis from α-acetobromoglucose, it was nevertheless regarded as reasonable to assume that the glycoside, and therefore adenosine, had the β-configuration.

Similarly the identity of the dialdehydes produced by the periodate oxidation of cytidine and cytosine-3-β-D-glucopyranoside, and of uridine and uracil-3-β-D-glucopyranoside[74] also demonstrated the β-configuration of the natural nucleosides and confirmed the location of the glycosidic linkage.

The slight element of doubt concerning the β-configuration of the nucleosides caused by the synthetic method used for the glycosides described above was removed by the synthesis of adenine-9-β-D-mannopyranoside (*XXI*) by a completely unambiguous method, which on oxidation with periodate gave the identical dialdehyde (*XIX*) to that obtained from adenosine (*XVIII*)[75].

Further proof of the β-configuration of the natural ribonucleosides was afforded by Clark, Todd and Zussman[76], who by the toluene-*p*-sulphonylation of 2′,3′-isopropylidene adenosine prepared the toluene-*p*-sulphonyl ester, 2′,3′-isopropylidene-5′-toluene-*p*-sulphonyladenosine (*XXII*). On heating in acetone solution this ester is rapidly converted into an ionic isomer which yields an ionic iodide on treatment with sodium iodide. Chemical studies of the ionic compound together with x-ray crystallographic analysis showed it to be the salt of 2′,3′-isopropylidene-$N_{(3)}$,$C'_{(5)}$-cycloadenosine (*XXIII*) in which a new ring has been formed by intramolecular alkylation. From steric considerations the formation of this cyclonucleoside is only possible if the original nucleoside possessed the β-configuration. Similar cyclo-derivatives are not produced by the toluene-*p*-sulphonyl derivatives of inosine and uridine, but 2′,3′-isopropylidene-5′-toluene-*p*-sulphonylcytidine rearranges to give a cyclo-derivative, 2′,3′-isopropylidene-$O_{(2)}$,5′-cyclocytidine (*XXIV*) in a similar manner.



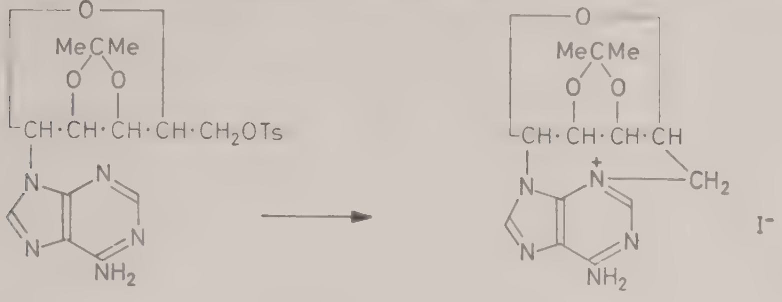
(*XVIII*) Adenosine

(*XX*) Adenine-9-β-D-glucopyranoside

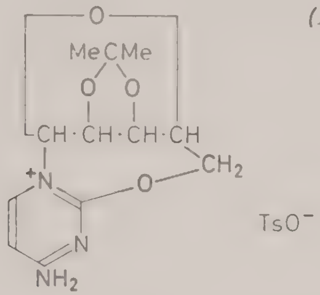
(*XIX*) Dialdehyde

(*XXI*) Adenine-9-β-D-mannopyranoside

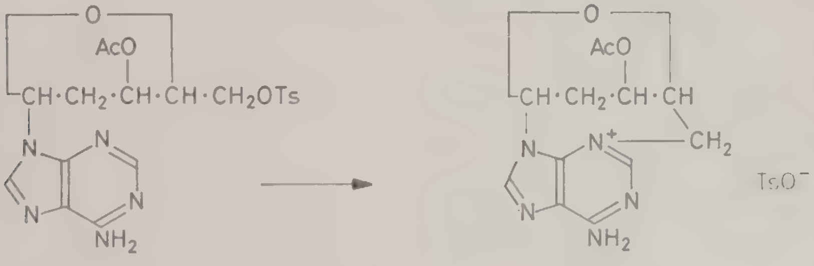
The above method is of particular importance since it may be applied to the deoxyribonucleosides, to which the periodate method, for obvious reasons, is not applicable. The method has been adopted by Andersen, Hayes, Michelson and Todd[1] to prove the β-configuration of deoxyadenosine and deoxycytidine. Toluene-*p*-sulphonylation of 3′-acetyl-2′-deoxyadenosine yields 3′-acetyl-2′-deoxy-5′-toluene-*p*-sulphonyladenosine (*XXV*) which is readily converted into the cyclonucleoside (*XXVI*) salt in hot acetone.



(*XXII*) (*XXIII*)



(*XXIV*)



(*XXV*) (*XXVI*)

The $O_{(2)}$,3′-cyclothymidine and $O_{(2)}$,5′-cyclothymidine cyclo-derivatives have been prepared by Michelson and Todd[77] from thymidine via the corresponding methanesulphonyl- or toluene-*p*-sulphonylthymidines. The chemical behaviour of the cyclothymidines is in accord with the β-configuration of thymidine. The β-configuration has also been confirmed by the x-ray crystallographic examination of 5′-bromo-5′-deoxythymidine[66]. No examination of the configuration of the glycosidic linkage in deoxyguanosine has been carried out, but in view of the proven β-configuration of deoxyadenosine, deoxycytidine and thymidine, Andersen, Hayes, Michelson and Todd[1] consider it virtually certain that all the natural deoxyribonucleosides will have the β-configuration.

The structures of cytidine[64] and of 5′-bromo-5′-deoxythymidine[66] have been determined by x-ray methods and preliminary studies have been made on adenosine, guanosine and uridine[73] and on thymidine and deoxycytidine[20]. The structure of cytidine, as given by Furberg[64], is shown in *Figure 24* and confirms the furanose structure of the D-ribose, the location of the sugar residue on $N_{(3)}$, and the β-configuration of the glycosidic linkage. These structural

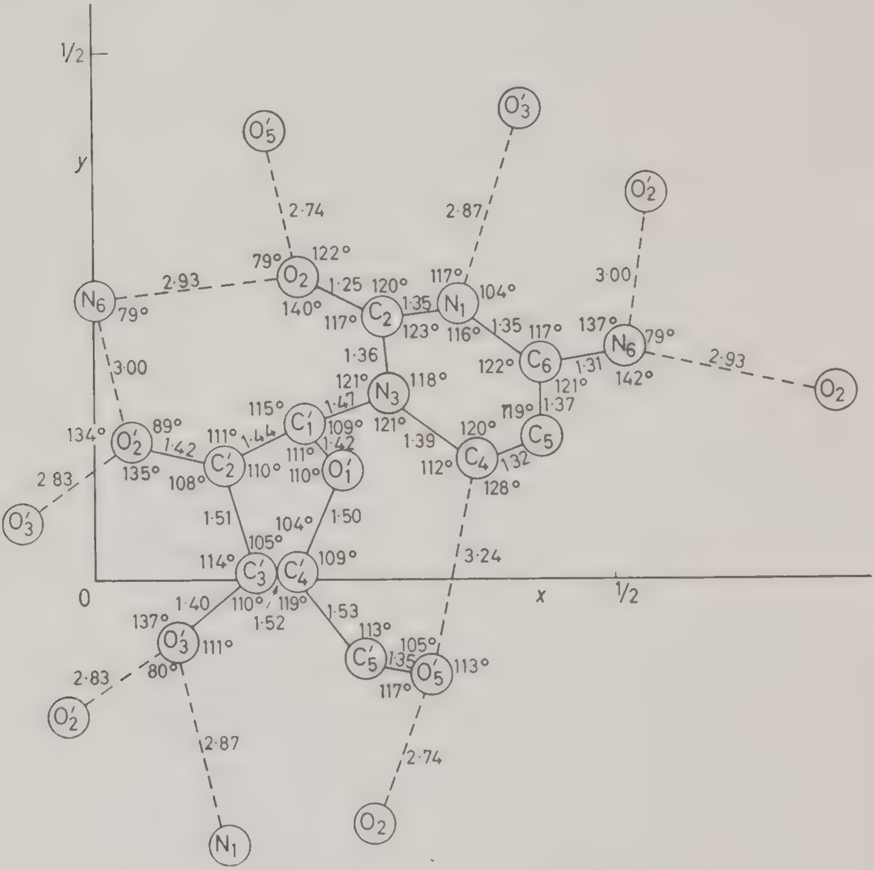

½
y
O′5
O′3
O′2
2·74
2·87
3·00
N6
2·93
O2
79°
122°
1·25
120°
117°
N1
104°
140°
C2
1·35
123°
116°
137°
79°
3·00
1·36
121°
N3
118°
C6
122°
1·31
121°
142°
115°
1·47
C′1
109°
121°
C5
1·37
119°
134°
O′2
89°
1·42
111°
1·44
1·39
120°
C4
112°
1·32
128°
135°
C′2
110°
O′1
2·83
108°
1·51
104°
1·50
3·24
105°
114°
C′3
C′4
109°
0
1·40
110°
119°
1·53
x
137°
1·52
113°
O′3
111°
105°
C′5
1·35
O′5
113°
80°
117°
N1

Figure 24. The structure of cytidine
(S. Furberg: *Acta Cryst.* 1950, **3**, p. 329)

details have also been confirmed for thymidine by the determination of the structure of the 5′-bromo-5′-deoxy derivative by Huber[66] (*Figure 26*). Other information, of importance to the structure of nucleic acids, is also obtained from these structures. The six atoms of the pyrimidine rings lie in the same plane, in agreement with the observations of Clews and Cochran[21, 22] and of Pitt[24]. The $C'_{(1)}$, $O'_{(1)}$, $C'_{(2)}$ and $C'_{(4)}$ atoms of the ribose and deoxyribose residues lie almost in one plane, but the $C'_{(3)}$ atom is out of this plane by, in the case of ribose, about 0·5 Å. The $N_{(3)}$—$C'_{(1)}$ bond has a length in cytidine of 1·47 Å and in the thymidine derivative of 1·55 Å, and is therefore a single bond in both cases. The angles between the bonds are such (see *Figures 24* and *25*) that the pyrimidine ring is oriented almost perpendicularly to the main plane of the sugar in both the nucleosides. This observation is of considerable importance to the structure of nucleic acids, particularly in view of the earlier assumption that the two rings were parallel.

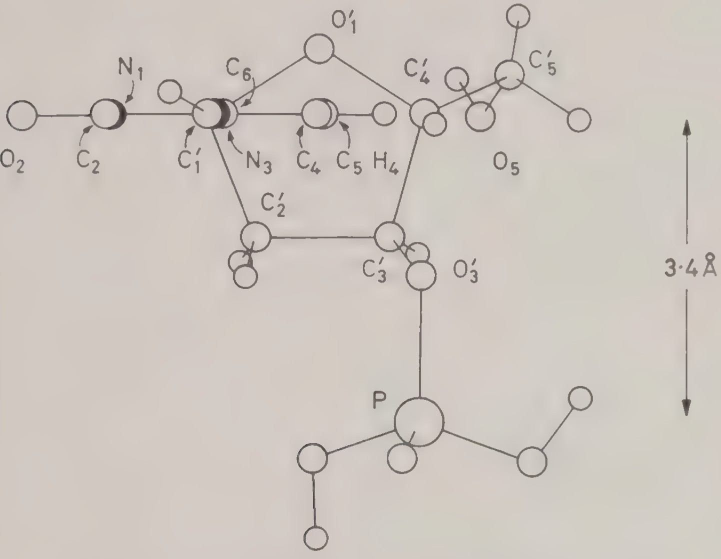


Figure 25. The structure of a pyrimidine nucleotide
(S. Furberg: *Acta chem. scand.* 1952, **6**, p. 635)

From the resemblance of the cell dimensions of uridine to those of cytidine[73], it was concluded that the stereochemistry of the two molecules is similar. The results on adenosine[73] were not sufficient to give a molecular projection as was obtained for cytidine, but they nevertheless were sufficient to confirm the position and configuration of the glycosidic linkage. From the similarity of the cell dimensions Furberg considers that guanosine has a similar structure to adenosine.

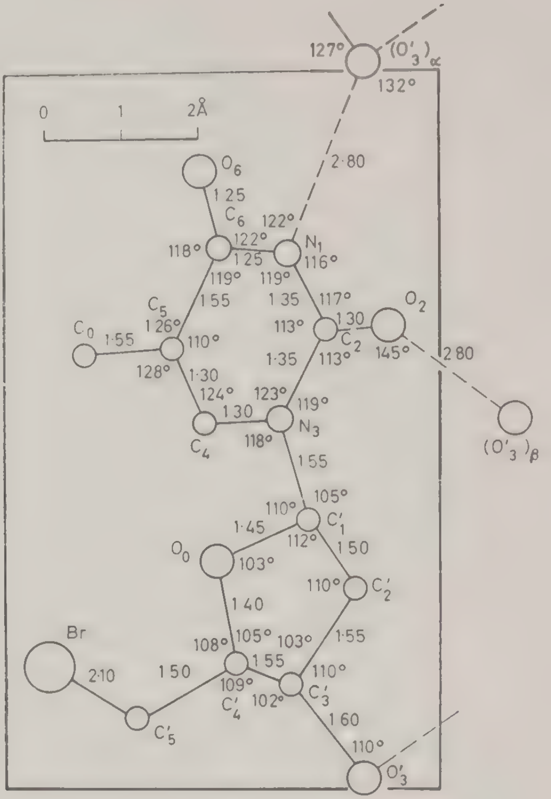

0
1
2Å
O6
1·25
C6
122°
122°
118°
1·25
N1
116°
119°
119°
1·55
1·35
117°
O2
C5
1·26°
113°
1·30
C0
1·55
110°
C2
145°
2·80
1·35
113°
128°
1·30
124°
123°
119°
1·30
N3
C4
118°
1·55
(O′3)β
105°
110°
C′1
1·45
112°
1·50
O0
103°
110°
C′2
1·40
Br
108°
105°
103°
1·55
2·10
1·50
1·55
110°
109°
C′4
102°
C′3
C′5
1·60
110°
O′3
127°
(O′3)α
132°
2·80

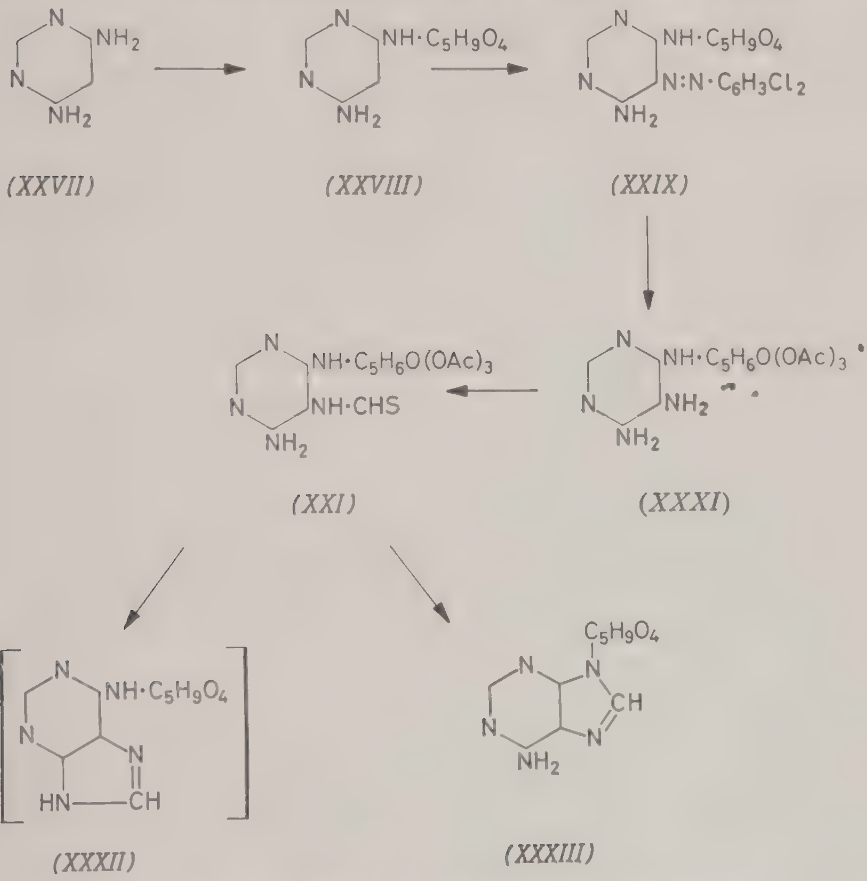
Figure 26. The structure of 5′-bromo-5′-deoxythymidine (M. Huber: *Acta Cryst.* 1957, **10**, p. 131)

THE SYNTHESIS OF NUCLEOSIDES[78]

The first synthesis of purine and pyrimidine nucleosides was made by Emil Fischer and his co-workers in 1914[79–82] by the interaction of suitably substituted purines and pyrimidines with acetohalogeno-sugars. This method was later used both by Levene[83–85] and by Hilbert and their collaborators[86–89] for the synthesis of a variety of purine and pyrimidine glycosides. None of these workers was successful, however, in synthesizing a natural nucleoside) the nearest approach being that of Hilbert and Rist[89] who synthesized 3-D-ribopyranosidouracil. Successful syntheses of all the ribonucleosides and the pyrimidine deoxyribonucleosides have been effected since 1944 by Todd and his collaborators.

Synthesis of purine ribonucleosides

Attempts to synthesize the purine nucleosides have followed two general methods. The first type of synthesis involved the preparation of a glycoside of a pyrimidine or an iminazole with subsequent completion of the purine nucleus by building on the second ring. This method[90, 91] is a modification of the general synthesis, introduced by Traube, for the synthesis of adenine. 4,6-Diaminopyrimidine (*XXVII*) is condensed with a pentose (or a hexose) by heating in ethyl alcohol with an acid catalyst under conditions which allow continuous removal of the water liberated in the reaction[92] to give the appropriate 6-amino-4-glycosidaminopyrimidine (*XXVIII*). This is now coupled with the diazonium salt derived from *p*-chloroaniline or 2,5-dichloroaniline in neutral or weakly alkaline solution to give the crystalline azo-compound (*XXIX*)[93]. The sugar hydroxyl groups are acetylated and the azo group reduced to amino to give acetylated 4,5-diamino-6-glycosyl-


(*XXVII*) (*XXVIII*) (*XXIX*)

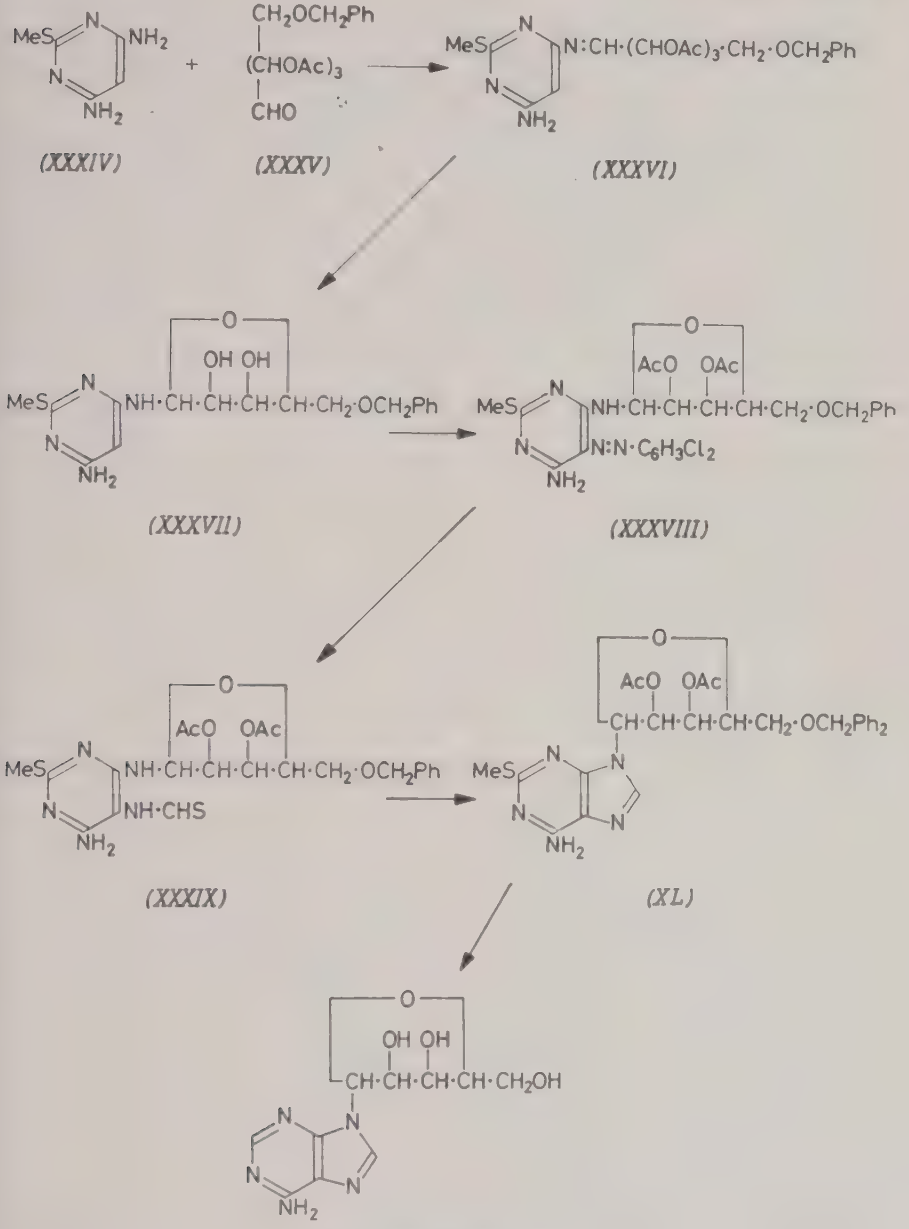
(*XXI*) (*XXXI*)

(*XXXII*) (*XXXIII*)

aminopyrimidine (*XXX*) which is converted into the corresponding 5-thioformamidopyrimidine (*XXXI*) by reaction with aqueous sodium dithioformate. Cyclization occurs on heating in dry pyridine to give a mixture of the 6-glycosidaminopurine (*XXXII*) and the required 9-glycosidoadenine (*XXXIII*)[94]. These two products are readily separated, by the solubility of the former and the insolubility of the latter in alkali, and distinguished by the ready deamination of the latter compound to the corresponding 9-glycosidohypoxanthine. Deacetylation can occur before or after separation. The yield of purine glycoside obtained in this synthesis may be improved by cyclization of the thioformamidopyrimidine in alcoholic alkoxide solutions[95]. This general method of synthesis has been used for the preparation of 9-D-xylopyranosyl-[96], 9-D-ribopyranosyl-[97], 9-D-mannopyranosyl-[75], and 9-D-glucopyranosyladenine[98], 9-D-xylopyranosyl-2-methylthioadenine[99] and 9-D-glucopyranosylisoguanine[100]. There can be no doubt that in compounds prepared by this synthesis, the glycosyl residue is located at the 9- position. The β-configuration of this linkage is based on theoretical reasoning and optical evidence. The above compounds are therefore all 9-β-glycosides[74, 75, 101].

The above method of synthesis yields glycosides with a pyranose structure. All the natural ribonucleosides, however, possess the furanose structure and thus, in order to effect their synthesis, the method requires modification. Kenner, Taylor and Todd[102] accomplished the synthesis of adenosine by condensing in methyl alcohol containing ammonium chloride, 2,3,4-triacetyl-5-benzyl-D-ribose (*XXXV*) with 4,6-diamino-2-methylthiopyrimidine (*XXXIV*) to give a Schiff base (*XXXVI*). The presence of the 5-benzyl group in the aldehydo-sugar is necessary to prevent furanose–pyranose conversion until the glyoxaline ring of the purine had been closed[103, 104]; the 2-methylthio group is necessary in order to increase the reactivity of the aminopyrimidine. The Schiff base is deacetylated with methanolic ammonia to give the glycoside (*XXXVII*) and coupled with diazotized 2,5-dichloraniline. The crude azo-compound is acetylated (*XXXVIII*), purified by chromatography, reduced with zinc dust and acetic acid and the resulting 5-amino compound directly thioformylated. The thioformamidoglycoside (*XXXIX*) is cyclized using sodium methoxide to give the purine glycoside (*XL*). The 2-methylthio and 5′-benzyl groups are simultaneously removed by hydrogenolysis with Raney nickel, and deacetylation of the product yields adenosine (9-β-D-ribofuranisoadenine) (*XLI*), identical with the natural nucleoside from yeast ribonucleic acid.

The synthesis of the purine nucleosides starting from iminazoles in place of purines has also been attempted[105, 106]. The method is


MeS
NH₂
CH₂OCH₂Ph
(CHOAc)₃
CHO
N:CH·(CHOAc)₃·CH₂·OCH₂Ph
OH OH
NH·CH·CH·CH·CH·CH₂·OCH₂Ph
AcO OAc
N:N·C₆H₃Cl₂
NH·CHS
CH·CH·CH·CH·CH₂·OCH₂Ph₂
CH·CH·CH·CH·CH₂OH

(XXXIV) (XXXV) (XXXVI)

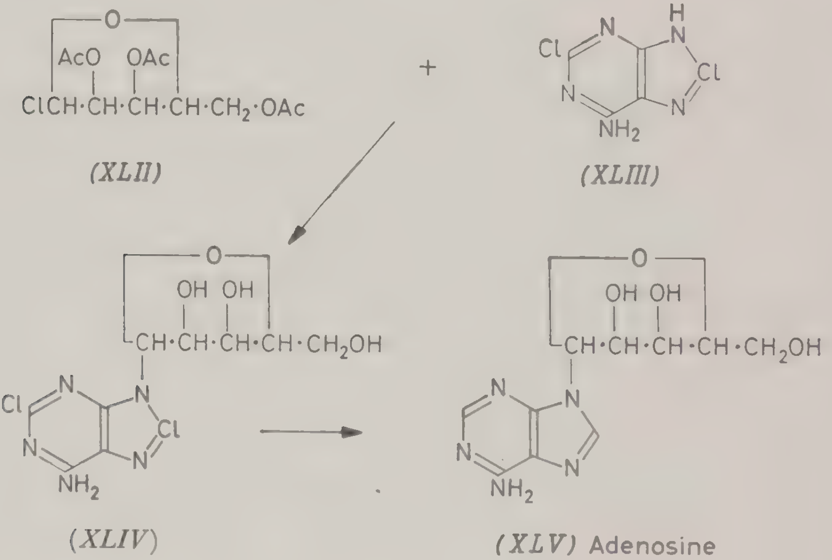
(XXXVII) (XXXVIII)

(XXXIX) (XL)

(XLI) Adenosine

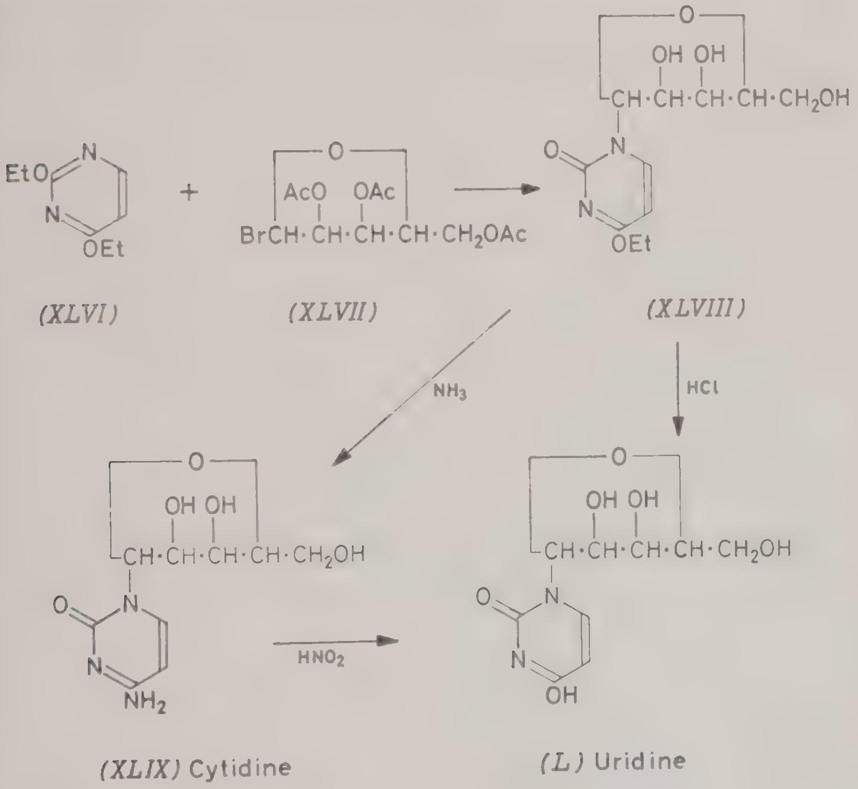
largely restricted to the xanthine glycosides and xanthosine has been synthesized in this way[107].

The second general method of synthesis, developed by Davoll, Lythgoe and Todd[108], is a modification of the original method of Fischer and Helferich[79] in which a silver salt of the appropriately substituted purine was reacted with an acetohalogeno-sugar. To apply this method to the synthesis of the natural ribonucleosides an acetohalogenoribofuranose is required. This was prepared by the method of Howard, Lythgoe and Todd[109]; 1,2,3-triacetyl-5-trityl-D-ribose, first prepared by Bredereck, Köthnig and Berger[110] is converted into 1,2,3-triacetyl-D-ribofuranose, by removing the trityl group by hydrogenolysis, which in turn is converted by acetylation into 1,2,3,5-tetra-acetyl-D-ribofuranose. This is then converted into the crude syrupy acetochloro-D-ribofuranose (*XLII*) by treatment with dry ethereal hydrogen chloride. Reaction with 2,8-dichloroadenine silver (*XLIII*) and deacetylation of the product gives 2,8-dichloro-9-β-D-ribofuranoside (*XLIV*). Hydrogenation of (*XLIV*) in aqueous sodium hydroxide in the presence of palladized barium sulphate, gives 9-β-D-ribofuranosidoadenine (*XLV*) identical with adenosine from yeast ribonucleic acid. Guanosine has been prepared by a similar method[111].



Synthesis of pyrimidine ribonucleosides

The method employed for the synthesis of pyrimidine nucleosides is analogous to the second method described for the synthesis of purine nucleosides and involves the reaction between an aceto-halogeno-sugar and a suitably substituted pyrimidine. The early investigators employed pyrimidine derivatives containing substituents such as hydroxyl, to which, as a result of their capacity for prototropic change, the glycosyl residue became attached. This difficulty was overcome by Hilbert and his collaborators by employing 2,6-dialkoxypyrimidines in which tautomerization possibilities are excluded[86, 87]. Interaction of acetobromo-D-ribofuranose (*XLVII*) with 2,6-diethoxypyrimidine (*XLVI*)[109] gives a product (*XLVIII*) which on treatment with methanolic ammonia yields a mixture of bases from which cytidine (*XLIX*) may be isolated by recrystallization and which is identical with natural cytidine. Deamination yields uridine (*L*) (alternatively treatment of the product from the initial reaction with hydrochloric acid will give uridine). This synthesis of cytidine by Howard, Lythgoe and Todd[109] was the first synthesis of a natural nucleoside.

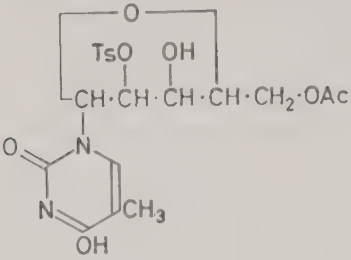

EtO
N
N
OEt
+
O
AcO OAc
BrCH·CH·CH·CH·CH₂OAc
O
OH OH
CH·CH·CH·CH·CH₂OH
O
N
N
OEt
NH₃
HCl
O
OH OH
CH·CH·CH·CH·CH₂OH
O
N
N
NH₂
HNO₂
O
OH OH
CH·CH·CH·CH·CH₂OH
O
N
N
OH

(XLVI) *(XLVII)* *(XLVIII)*

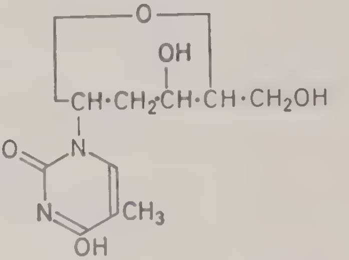
(XLIX) Cytidine *(L)* Uridine

Synthesis of deoxyribonucleosides

The synthesis of thymidine and of 2′-deoxyuridine, the natural pyrimidine nucleosides of deoxyribose nucleic acid, has recently been described[112, 113]. The 3-β-D-ribofuranosyl thymine (or uracil derivative) is converted into the 5′-acetate through the 2′,3′-isopropylidene derivative. Toluene-*p*-sulphonylation yields 5′-acetyl-2′-toluene-*p*-sulphonyl-ribosylthymine (*LI*) which on treating with sodium iodide yields the 2′-deoxy-2′-iodo compound which on hydrogenation over a palladium catalyst and deacetylation gives thymidine (*LII*).



(*LI*)



(*LII*)

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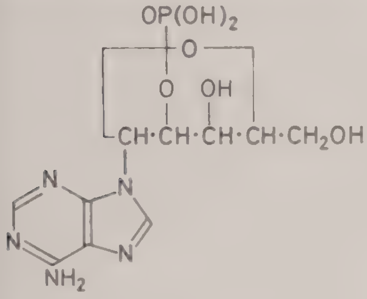
6

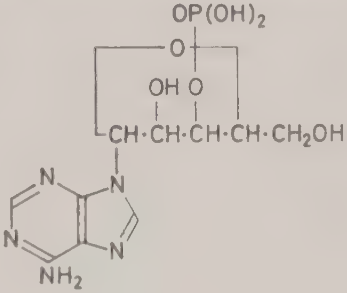
THE STRUCTURE AND SYNTHESIS OF THE NUCLEOTIDES

INTRODUCTION

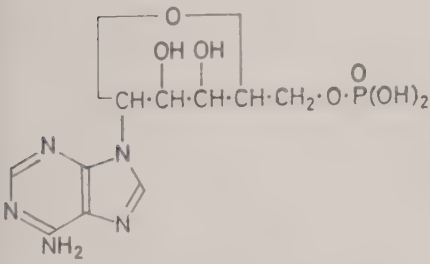
THE nucleotides are the phosphoric esters of the nucleosides. The monomeric structural units of the nucleic acids are the monophosphoric esters of the nucleosides. Diphosphates may arise either by the phosphorylation of a second sugar hydroxyl group by a second phosphoric acid molecule, or by the formation of a pyrophosphate. Triphosphates generally contain one or two pyrophosphate linkages. In the ribonucleotides, the monophosphates of the



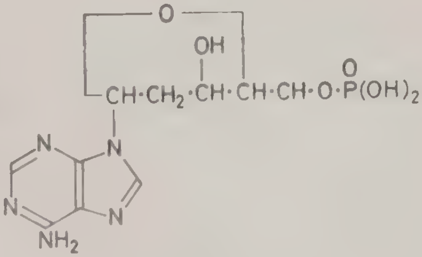
(I)
Adenosine-2′-phosphate
Adenylic acid *(a)*



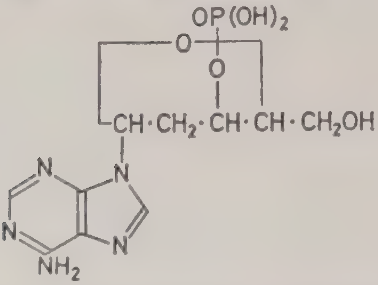
(II)
Adenosine-3′-phosphate
Adenylic acid *(b)*



(III)
Adenosine-5′-phosphate
Muscle adenylic acid



(IV)
Deoxyadenosine-5′-phosphate

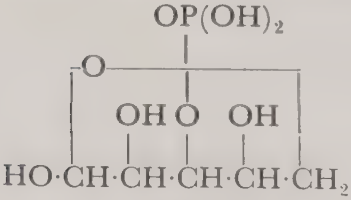


(V) Deoxyadenosine-3′-phosphate

ribonucleosides, the phosphoric acid group may be situated on the 2′-, 3′- or 5′- position of the sugar residue. In the deoxyribonucleotides, however, only the 3′- and 5′- positions are available. All these isomeric modifications have been isolated from the chemical or enzymic hydrolysates of nucleic acids. The structures of the five known adenine nucleotides are given as examples.

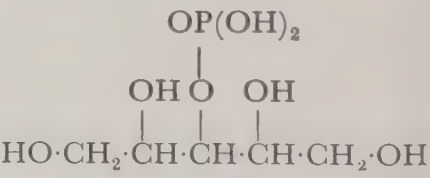
THE ISOMERISM OF THE RIBONUCLEOTIDES

For many years it was generally accepted that only four separate nucleotides could be isolated from the chemical hydrolysates of yeast ribonucleic acid. The position of the phosphoric acid group on yeast adenylic acid was studied by Levene and Harris[1-3], who established its location on the sugar residue rather than on the purine ring by deaminating adenylic acid to an inosine phosphate which on hydrolysis readily gave hypoxanthine and a ribose phosphate[3]. This ribose phosphate differed from ribose-5-phosphate and the possible structures for adenylic acid were thus narrowed to adenosine-2′- and adenosine-3′-phosphate (*I* and *II*). Catalytic reduction of the ribose phosphate yielded[2] an optically inactive ribitolphosphate (*VII*) which would indicate, if the adenylic acid were a single substance and if no migration of phosphate groups had occurred during the hydrolysis and reduction, that the ribose phosphate was ribose-3-phosphate (*VI*) and that the original adenylic acid was adenosine-3′-phosphate.



(*VI*)

Ribose-3-phosphate



$$\mathrm{HO{\cdot}CH_2{\cdot}\overset{OH}{\overset{|}{C}H}{\cdot}\overset{OP(OH)_2}{\overset{|}{\overset{O}{\overset{|}{C}H}}}{\cdot}\overset{OH}{\overset{|}{C}H}{\cdot}CH_2{\cdot}OH}$$

(*VII*)

Ribitol-3-phosphate

The ribose phosphate from guanylic acid was considered to be identical with that from adenylic acid ; guanylic acid was thus also regarded as a -3′-phosphate[1]. By analogy the pyrimidine nucleotides were assumed to be -3′-phosphates also. An attempt to confirm these conclusions by an unambiguous synthesis of the nucleoside-3′-phosphates was made by Michelson and Todd[4], who obtained adenylic and uridylic acids identical with the natural products. However, the synthetic route adopted involved as intermediates the benzylidene nucleosides which had been formulated incorrectly as 3′,5′-benzylidene derivatives[5, 6]. These derivatives have now been shown by Brown, Haynes and Todd[7] to be in fact the 2′,3′-benzylidene derivatives. The nucleotides synthesized by Michelson and Todd[4] cannot therefore be regarded definitely as the -3′-phosphates, since they may equally well have been -2′-phosphates. The benzylidene derivatives have been used to prepare, by direct phosphorylation, nucleotides[4, 8, 9] which were originally formulated as -2′-phosphates, but which must now be regarded as -5′-phosphates.

As described in Chapter 4, the chemical hydrolysate of ribonucleic acid contains, in fact, not four separate nucleotides, but eight. These eight nucleotides are made up of four sets of two isomers termed by Carter and Cohn[10] the *a* and *b* isomers. Thus adenylic acid prepared from the chemical hydrolysate of ribonucleic acid is not a single substance, as originally thought, but a mixture of the adenylic acids, *a* and *b*. These isomers may be separated and isolated by ion-exchange and paper chromatography and paper electrophoresis (see Chapter 4). The precise determination of the structures of the isomeric nucleotides is necessary for the rigid definition of the position of the internucleotide linkage in the nucleic acids, and an extensive study has been made of the isomers culminating in the unambiguous synthesis of adenylic acid *a* and of uridylic acid *a*.

Carter and Cohn[10] degraded the isomeric adenylic acids *a* and *b* to adenine and adenosine and suggested that they were the adenosine-2′- and adenosine-3′-phosphates. The possibility that one of the isomers was adenosine-5′-phosphate was excluded by Carter and Cohn on enzymic and chemical evidence and this was confirmed by Brown, Haynes and Todd[7] who demonstrated that both isomers are stable to periodic acid oxidation whereas adenosine-5′-phosphate is not.

The difference in the position of the phosphate group is not the only form of isomerism possible. Carter[11] suggested the alternative possibility that both acids might be -3′-phosphates and that they might differ by virtue of α-β-isomerism at the glycosidic linkage.

This possibility appeared to be favoured by the work of Doherty[12], who repeated the work of Levene and Harris[2] on both adenylic acids (as well as on the guanylic acid isomers) by preparing ribitolphosphates from the *a* and *b* isomers. Both were found to be optically inactive which was regarded as evidence that both were derived from a -3′-phosphate. However, only one adenosine, having the β-configuration, is isolated from nucleic acids whereas if the *a* and *b* isomers possessed different configurations at the glycoside linkage the isomerism of the nucleotides should be reflected by an isomerism of the nucleosides. Moreover, the *a* and *b* isomers readily undergo interconversion in dilute acidic solutions to give an equilibrium mixture of the two isomers[13, 14]. Such an interconversion is unlikely to result from an α-β-isomerization since in that case one form, being more stable, would predominate and isomerization would be accompanied by furanose–pyranose changes which are not observed.

On the basis of these arguments, Brown and Todd[13] considered that the possibility of the isomerism arising from different configurations of the glycosidic linkage is most improbable. This view was substantiated by Brown and Todd[13] by the synthesis of a mixture of adenylic acids *a* and *b*. This was accomplished by the phosphorylation of 5′-trityl adenosine with dibenzyl chlorophosphonate. Removal of the trityl and benzyl groups by acetic acid and hydrogenation yielded a mixture of two adenylic acids. These two acids were separated by paper and ion-exchange chromatography and identified as adenylic acid *a* and adenylic acid *b* by comparing their ion-exchange and paper chromatographic behaviour, x-ray powder photographs and infra-red spectra with the natural adenylic acid isomers isolated from yeast ribonucleic acid. On the basis of this evidence, it would appear certain that the adenylic acid *a* and *b* isomers are the adenosine-2′- and -3′-phosphates, although not necessarily respectively. It would also appear reasonable to assume that the isomerism was of the same type in the other nucleotides. A similar conclusion was reached by Loring, Hammell, Levy and Bortner[15] for the cytidylic acids *a* and *b* on the grounds that an identical nucleoside, *viz.* cytidine, was produced on enzymic dephosphorylation.

The remaining problem was to identify adenylic acid *a* as the -2′-, or -3′-phosphate or solve the similar problem for the other nucleotide isomers. Cavalieri[16] determined the densities of solutions of the *a* and *b* isomers of cytidylic and adenylic acids and found that the *b* isomer has the greater density at all concentrations. Since, as the distance between the charged groups of a zwitterion is increased, the density of its aqueous solution increases[17] (because of increased electrostriction) it is concluded that the *b* isomer possesses the greater separation of charge. From the known structure of cytidine (*Figure 24*, Chapter 5), this would make the *b* acid the -3′-phosphate.

This view is substantiated by the pK_a' values of the respective amino groups. Those of the *a* and *b* isomers of cytidylic acid are 4·36 and 4·28 respectively and of adenylic acid, 3·81 and 3·74 respectively. The latter values are in reasonable agreement with those of Alberty, Smith and Bock[18] who gave the values as 3·80 and 3·65 respectively and of Kuna[19] who gave 3·79 and 3·56 respectively. On theoretical grounds, the pK_a' value will be lower for that isomer having the greater separation of charge[20], *i.e.* for the -3′-phosphate. The ionization of the sugar hydroxyl groups in the pH range 12–14 has also been studied[21] from the variation of the ultra-violet absorption spectrum with pH. On the assumption that it is the 2′-hydroxyl group dissociating in this range, it was considered that the results confirmed the above conclusions. Another attempt to study the problem involved the hydrolysis of adenylic acids *a* and *b* with a sulphonic acid resin. However, since either acid gave a mixture of ribose-2- and -3-phosphates it is clear that migration of the phosphate group is occurring[22].

Further evidence for the identity of the *a* and *b* isomers with the -2′- and -3′-phosphates came from a comparison of the infra-red spectrum of deoxycytidine-3′-phosphate with that of cytidylic acids *a* and *b*[23]. The similarity of the spectrum of deoxycytidine-3′-phosphate with that of the *b* isomer, and its difference from that of the *a* isomer, again gave strong support to the identity of the *b* isomer and the -3′-phosphate.

Although there is agreement between all the evidence given above concerning the structure of the isomeric nucleotides, it is, however, circumstantial and rigid confirmation by chemical synthesis is desirable. This has been supplied by the synthesis by Brown, Fasman, Magrath and Todd[24] of adenosine-2′-phosphate, and by Brown, Todd and Varadarajan[25] of uridine-2′-phosphate. The synthesis of adenosine-2′-phosphate was carried out as follows. Acetylation of 5′-acetyladenosine[7] leads to a mixture of mono-, di- and triacetyladenosines from which one, and *only* one, crystalline diacetyladenosine could be isolated. This diacetyladenosine was phosphorylated with O-benzyl phosphorous OO-diphenyl phosphoric anhydride, yielding a diacetyladenosine benzyl phosphite which, by the action of N-chlorosuccinimide followed by very mild hydrolysis with aqueous pyridine, gave diacetyladenosine benzyl hydrogen phosphate. Hydrogenation and deacetylation gave the nucleotide.) The final product was identified by comparison with an authentic specimen as adenylic acid *a*[26].

The significant point of this synthesis is that *only* adenylic acid *a* is produced, there being no trace of the *b* isomer. The structure of the *a* isomer thus hinges on the structure of the original diacetyladenosine, since the vacant position must correspond to the *a* group. If the position of the phosphate group in the ribofuranose residue in adenylic acid *a* is denoted by *a*, and that in the *b* isomer by *b*, then it follows that the diacetyladenosine used was *b*,5′-di-acetyladenosine. This on treatment with toluene-*p*-sulphonyl chloride followed by deacetylation gives *a*-toluene-*p*-sulphonyladenosine. Methylation followed by removal of the toluene-*p*-sulphonyl group and acid hydrolysis yielded a mixture of methylated sugars. Chromatography showed these to be a mixture of 5-methyl-, 3-methyl- and 4-methylribose and 3,5-dimethylribose. The complete absence of any derivative with a methyl group in the 2- position can only indicate that the *a*-toluene-*p*-sulphonyladenosine was in fact 2′-toluene-*p*-sulphonyladenosine, *i.e.* that the 2′- position is equivalent to the *a* position. It thus follows that adenylic acid *a* is adenosine-2′-phosphate and hence adenylic acid *b* is adenosine-3′-phosphate.

The synthesis of uridylic acid *a*[25] followed a similar synthetic course, but the proof of the structure of the corresponding diacetyluridine was complicated by the formation on deacetylation of the diacetyl-toluene-*p*-sulphonyluridine of an $O_{(2)}$,2′-cyclouridine, which on hydrolysis yielded a new nucleoside identified as 3-β-D-arabofuranosyluracil. The identity of uridylic acid *a* with uridine-2′-phosphate also proves that cytidylic acid *a* is cytidine-2′-phosphate. This conclusion had been reached somewhat earlier by Baron and Brown[27], who found that fission of cytidylic acid *a* and *b* by hydrazine yielded ribose-2- and ribose-3-phosphates respectively, there being no migration of the phosphate during this procedure.

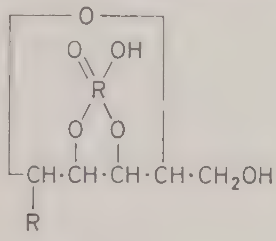
The Ribonucleoside-5′-phosphates

The ribonucleoside-5′-phosphates are obtained from ribonucleic acids by enzymic hydrolysis as described in Chapter 4.

The structures of these nucleotides have been relatively easy to determine. In general they have either been compared directly with the corresponding synthetic nucleotide or, in the case of adenosine-5′-phosphate (muscle adenylic acid) converted, by deamination, into inosine-5′-phosphate. The structure of the latter nucleotide was determined by Levene and Jacobs[28] by hydrolysing with acid to give the ribose-5-phosphate, which was identified by oxidation with nitric acid to give a ribonic acid phosphate and not a ribo-trihydroxyglutaric acid phosphate. The ribose phosphate was also identified by comparison with synthetic ribose-5-phosphate[29]. The acids are also oxidized by periodate consuming 1 mole[30].

The Cyclic Phosphates

The discovery of the *a* and *b* nucleotides and their identification as the -2′- and -3′-phosphates, together with the discovery of their ready interconvertibility in dilute acid solution, suggested to Brown and Todd[13] that this interconversion proceeded through a cyclic nucleotide (*VIII*) which could be formed from either a -2′- or -3′-phosphate. It was also suggested that during the alkaline hydrolysis of ribonucleic acid the corresponding cyclic phosphates would also be formed (see Chapter 8) which would be triesters, since the 5′- position is presumably joined to the adjacent nucleotide in the polynucleotide. These cyclic nucleotides have since been identified by Markham and Smith[31] in the hydrolysates of ribonucleic acid produced by the action of alkali and ribonuclease.



(*VIII*)

R = purine or pyrimidine

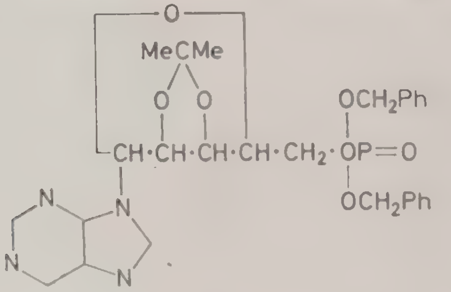
The cyclic nucleotides (*VIII*) (which are nucleoside-2′,3′-hydrogen phosphates) of adenosine, cytidine and uridine have been synthesized by Brown, Magrath and Todd[32], and identified with the products isolated from ribonucleic acid. The pyrimidine cyclic nucleotides are readily converted into the *b* acid by the further action of ribonuclease[32, 33] and into the *a* and *b* isomers by the action of acid. The cyclic phosphates of adenosine and guanosine, however, while being readily hydrolysed to the *a* and *b* isomers by acid, are not attacked by ribonuclease[33].

THE DEOXYPENTOSE NUCLEOTIDES

The pyrimidine deoxyribonucleotides may be prepared by enzymic or chemical hydrolysis of deoxyribose nucleic acid, but the purine deoxyribonucleotides may only be prepared by enzymic digestion. In view of the 2′-deoxy group, the only isomeric forms that can exist are the -3′- and -5′-phosphates. The deoxyribonucleotides are generally prepared by enzymic digestion of deoxyribonucleic acid and the structure of the resulting nucleotides depends upon the nature of the enzyme used. As described in Chapter 4, the deoxyribonuclease of calf pancreas yields deoxyribonucleotides which are dephosphorylated by 5′-nucleotidase[34] and which behave on ion-exchange columns in a similar manner to nucleoside-5′-phosphates[35]. It is therefore concluded that this enzyme yields the deoxynucleoside-5′-phosphates. The deoxyribonuclease from calf spleen[36] yields deoxyribonucleotides which have been shown enzymically to be the -3′-phosphates[37]. A deoxyribonuclease which has been isolated from *Micrococcus pyogenes*[38, 39] also apparently gives the -3′-phosphates.

THE SYNTHESIS OF THE RIBONUCLEOTIDES

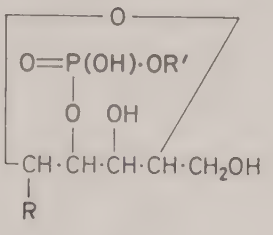
The synthesis of the -2′- and -3′-phosphates has already been fully described. The ribonucleoside-5′-phosphates were first synthesized by Levene and Tipson[40, 41], and more recently by Michelson and Todd[4], by the phosphorylation of the -2′,3′-isopropylidene derivatives with phosphoryl chloride in pyridine followed by removal of the isopropylidene group by acid hydrolysis. The yields obtained, however, were low and improvement in the nature of the phosphorylating agent was desirable. A more suitable reagent is dibenzyl phosphorochloridate[42]. Reaction of 2′,3′-isopropylidene adenosine with this reagent in pyridine gives the 5′-dibenzyl phosphate (*IX*); removal of the benzyl groups by catalytic hydrogenation and of the isopropylidene residue by acid hydrolysis yields adenosine-5′-phosphate[43]. Cytidine- and uridine-5′-phosphates have been prepared in this way[4].



(*IX*)

The Synthesis of the Cyclic Phosphates

The cyclic phosphates of adenosine, cytidine and uridine have been prepared from a mixture of the *a* and *b* isomers by reaction with trifluoroacetic anhydride[32]. The reaction most probably proceeds through the initial formation of a mixed anhydride (*X*) of the phosphate with trifluoroacetic acid. The mixed anhydride then acts as a phosphorylating agent towards the adjacent hydroxyl group, although it could conceivably react with another phosphate group to give a pyrophosphate, or with a hydroxyl group of another nucleotide to give a dinucleotide.



(*X*)

(R = purine or pyrimidine)
(R′ = $CO \cdot CF_3$)

THE SYNTHESIS OF THE DEOXYRIBONUCLEOTIDES

Thymidylic acid (thymine-5′-phosphate) and thymine-3′-phosphate have been synthesized by Michelson and Todd[44]. Phosphorylation of 5′-trityl thymidine with dibenzyl phosphorochloridate yielded, on hydrogenolysis of the product, thymidine-3′-phosphate.

Acetylation of 5′-trityl thymidine to 3′-acetyl-5′-trityl thymidine, and heating with acetic acid gave 3′-acetyl thymidine, phosphorylation with dibenzyl phosphorochloridate then yielded after removal of the protecting groups thymidine-5′-phosphate, identical with natural thymidylic acid. Similar methods have been used for the synthesis of deoxycytidine-[23], deoxyadenosine- and deoxyguanosine-3′- and -5′-phosphates[45]. The only difficulty that was experienced was the well-known lability of the purine deoxyribosides and their derivatives to acid, which necessitated some modification to the synthetic method. All the -5′-phosphates were found to be identical with those prepared from deoxyribose nucleic acid by enzymic hydrolysis.

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7

THE ACID–BASE PROPERTIES OF THE NUCLEIC ACID COMPONENTS

PURINES AND PYRIMIDINES

THE pK'_a values of some purines and pyrimidines are given in *Table 8.* Levene, Simms and Bass[1, 2] determined the dissociation constants of uracil and originally came to the conclusion that it possessed two acid dissociations with pK'_a values 9·28 and 13·36, which were attributed to the two —CO—NH— groups. Later, the same authors[3] corrected the original work and concluded that uracil possesses only one acid dissociation with a pK'_a value at 9·45. This conclusion and the pK'_a value have been confirmed by Taylor[4]. The weakness of the second dissociation is not entirely due to an electrostatic effect[5] and must be caused by a real difference in the two groups, although the values for 1- and 3-methyl uracil are very similar. This view is supported by the extreme weakness of the second dissociation of cytosine (which is the dissociation of the —NH—CO— group), pK'_a 12·16, compared with that of isocytosine (2-amino-6-hydroxypyrimidine), pK'_a 9·42, which suggests that the 2-carbonyl group promotes stronger basic properties in the —NH—CO— dissociation than does the 6-carbonyl group. From this and other similar evidence, differentiation has been made between the —N_1H—C_6O— and —N_3H—C_2O— dissociations in *Table 8.*

The introduction of the methyl group in the 5- position of uracil, as in thymine, has little effect on the pK'_a value of the —NH—CO— dissociation. The pK'_a values of cytosine and isocytosine, 4·60 and 4·01 respectively, can be ascribed to the amino group, the value of the dissociation constant being comparable with that of the aromatic amines: this view has been confirmed by titration in the presence of formaldehyde[4].

The dissociations of adenine and guanine of pK'_a values 4·15 and 3·3 respectively may be attributed to the amino groups and the guanine dissociation of pK'_a 9·2 will be due to the —NH—CO— group. These values are all comparable with those of the pyrimidines. The dissociation of adenine of pK'_a 9·8 and the dissociation of guanine of pK'_a 12·3 are almost certainly the dissociation of —NH— to —$\overline{\text{N}}$— in the imidazole ring. Benzimidazole has been found by Taylor[6] to have a basic association of pK'_a 5·3 and an acidic dissociation of pK'_a 12·3. These values must represent the dissociation of the —$\overset{+}{\text{N}}$H= and —NH— groups respectively.

Table 8. pK'_a values for purines and pyrimidines

	Amino —$\overset{+}{\text{N}}\text{H}^3$	—N_1H—C_6O—	—N_3H—C_2O—	*Imidazole* —NH—
Uracil		9·45[1-3]; 9·5[11]		
1-Methyl-uracil		9·99[3]		
3-Methyl-uracil		9·71[3]		
Thymine		9·82[3]; 9·9[11]		
Cytosine	4·6[3, 4]; 4·45[11]; 4·6[12]		12·16[3]	
5-Methyl-cytosine	4·8[12]; 4·6[11]		12·4[11]	
Isocytosine	4·01[3]	9·42[3]		
Adenine	4·15[6]; 4·12[13]; 4·16[14]; 4·22[16]			9·8[6,13]
Guanine	3·3[6]; 3·2[12]	9·2[6]; 9·6[12]; 9·36[15]		12·3[6]
Xanthine		7·7[12,17]		
1-Methyl-xanthine			7·7[6, 17]	12·05[6,17]
3-Methyl-xanthine		8·5[17]; 8·1[6]		11·3[6,17]
7-Methyl-xanthine		8·5[17]; 8·1[6]		
9-Methyl-xanthine		6·3[17]; 6·25[6]		
Hypo-xanthine		8·8[4]; 8·7[17]; 8·9[12,16]		12[4]

The value of the dissociation of benzimidazole of pK'_a 5·3 may be compared with that of imidazole, 7·1, obtained by Dedichen[7], whereas the value of the dissociation of pK'_a 12·3 is of the same order as that of the weakest dissociations of guanine, 1- and 3-methylxanthine and of hypoxanthine. As is to be expected, the corresponding dissociations are missing in 7- and 9-methylxanthine. The value for this dissociation in adenine, 9·8, is surprisingly low compared with the values for the corresponding groups in the comparable molecules cited above. Taylor[8] has ascribed the unusual acid strength of this group in adenine to the contribution to the resonance of the molecule of structures in which there is a negative charge on the nitrogen atoms in the pyrimidine ring and a positive charge on those in the imidazole ring. The positive charge on the imidazole nitrogens would tend to increase the acid strength of this group in the manner observed. Taylor considers that this explanation is supported by the greater length of the $C_{(4)}$—$C_{(5)}$ bond in adenine compared with that of the corresponding bond in guanine as shown by the crystallographic measurements of Broomhead[9] (see Chapter 5, p. 103). However, Cochran[10] considers that the resonance structures envisaged by Taylor would also make the bonds $C_{(9)}$—$C_{(8)}$ and $N_{(7)}$—$C_{(5)}$ single bonds and the bond $N_{(7)}$—$C_{(8)}$ a double bond whereas the crystallographic data indicate that all these bonds are similar and possess 20–50 per cent double bond character.

NUCLEOSIDES AND NUCLEOTIDES

The pK_a' values of the nucleosides and nucleotides are given in *Table 9*. The values for the nucleosides correspond closely to those of the parent purines and pyrimidines although there is a general tendency for them to be lowered, *i.e.* for the acid dissociations to be strengthened. An additional dissociation has been observed in most cases with a pK_a' value of between 12·3 and 12·5, which is of the correct order for the dissociation of a sugar hydroxyl group. The values for D-ribose have not been determined, but glucose has a pK_a' value of 12·1[18–20].

Comparison of the values for the nucleotides with those of the respective nucleosides, shows that the dissociation groups of the latter are supplemented by two additional dissociations having pK_a' values of 0·7–1·6 and 5·9–6·5. These are of the correct order for the first and second dissociations of a sugar phosphate. The values for glucose-3-phosphate are 0·84 and 5·67[21] and similar values have been obtained for other sugar phosphates[14, 22].

Since adenylic, guanylic and cytidylic acids are ampholytes containing the cationic amino and the anionic phosphate groups, there is the possibility that they will exist largely in the zwitterionic form. Calculations[5] show that adenylic and cytidylic acids exist in aqueous solution almost completely in the zwitterionic form but with guanylic acid the ratio of zwitterionic to uncharged form is only 7 : 1 because of the strong acidity of the amino group in the

Table 9. pK_a' values of the nucleosides and nucleotides

	Primary phosphoric acid	*Amino*	*Secondary phosphoric acid*	$—N_1H—C_6O—$	*Sugar hydroxyl*
Adenosine	—	3·45[1, 2]; 3·63[13]; 3·4[24]	—	—	12·5[1, 2]
Adenosine-2′-phosphate	0·89†	3·80[13]; 3·79[14]	6·15[13]; 6·21[14]	—	*
Adenosine-3′-phosphate	0·89†	3·65[13]; 3·56[14]; 3·70[1, 2, 25]	5·88[13]; 6·06[14]; 6·01†[1, 2]	—	*
Adenosine-5′-phosphate	*	3·74[13]; 3·89[14]; 3·8[26]	6·05[13]; 6·48[14]; 6·2[26]	—	*
Deoxyadenosine-5′-phosphate	*	4·4[27]	6·4[27]	—	*
Guanosine	—	1·6[1, 2]	—	9·2[1, 2]	12·3[1, 2]
Guanylic acid†	0·7†	2·3†[1, 2]; 2·49†[25]	5·92†[1, 2]; 6·05†[25]	9·38†[1, 2]; 9·33†[25]	*
Deoxyguanosine-5′-phosphate	*	2·9[27]	6·4[27]	9·7[27]	*
Cytidine	—	4·11[28]; 4·15[12]; 4·22[1, 2]	—	12·16‡[1, 3]	12·3[1, 2]
Cytidine-2′-phosphate	0·8†	4·30[12, 28, 29]; 4·36[24]	6·2[29]	—	*
Cytidine-3′-phosphate	0·8†	4·30[29]; 4·28[12, 24]; 4·24†[1, 2]; 4·22†[25]	6·0[29]; 5·97[1, 2]; 6·07†[25]	—	*
Cytidine-5′-phosphate	*	4·44[28]	*	—	*
Deoxycytidine-5′-phosphate	*	4·45[12]; 4·6[27]	6·6[27]	—	*
5-Methylcytidylic acid	*	4·4[12]	*	—	*
Uridine	—	—	—	9·17[1, 2]; 9·30[25]	12·5[1, 2]
Uridylic acid†	1·02†[1, 2]	—	5·88†[1, 2]	9·43†[1, 2]	*
Thymidylic acid	1·6[27]	—	6·5[27]	10·0[27]	*

*Not measured; †mixed *a* and *b* isomers; ‡$—N_3H—C_2O—$

protonated form. However, even here the zwitterionic form strongly predominates at the isoelectric point.

The values of the dissociation constants which have generally been listed in the past have been those of mixtures of the 2′- and 3′-phosphates. With the isolation of the separate isomers for each nucleotide, however, it has become possible to determine the pK_a' values for the individual isomers.

It is evident, from the values given in *Table 9*, that the introduction of the phosphate group has weakened the acid strength of the amino and the —NH—CO— dissociations. This, of course, is a mutual effect so that the secondary phosphate dissociation is strengthened compared with that in phosphoric acid (this will also be true for the primary dissociation, but this cannot be so accurately measured). This observation is in agreement with the field effect[23] and will be a function of the distance between the charged groups, the smaller this distance, the greater will be the shift of pK_a' values. This effect has been used by Cavalieri[24] in order to establish the configuration of the *a* and *b* isomers of the nucleotides as has been fully discussed in Chapter 6.

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8

THE STRUCTURE OF NUCLEIC ACIDS: 1 THE NATURE OF THE INTERNUCLEOTIDE BOND

INTRODUCTION

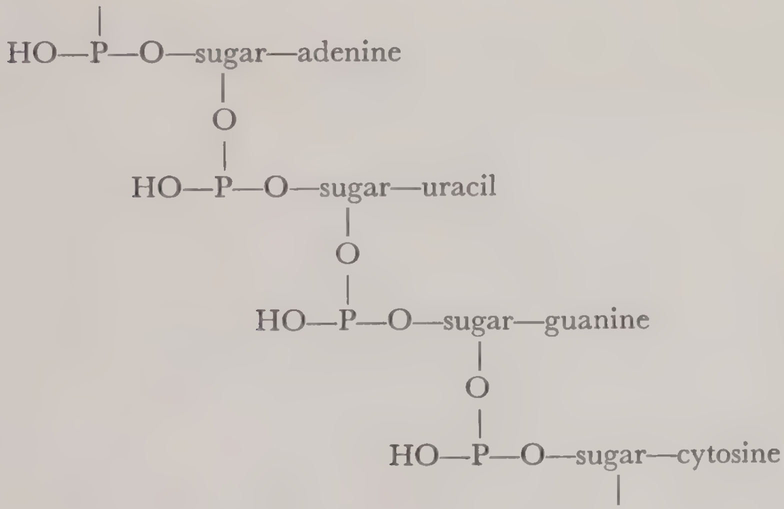
Now that all the essential structural features of the nucleotides have been established, it is possible to proceed to the consideration of the structure of the nucleic acids. From determinations of the molecular weight of nucleic acids, which will be described in Chapters 10 and 12, it is now recognized that all nucleic acids are macromolecules of molecular weight ranging from possibly only a few tens of thousand to several million. They are thus all polynucleotides. In establishing the structure of these polynucleotides, the first problem that must be considered is the nature and location of the primary bond that holds two adjacent nucleotides together in the polynucleotide.

THE INTERNUCLEOTIDE BOND IN PENTOSE NUCLEIC ACIDS

In the union of nucleotides to form a polynucleotide several types of linkage are theoretically possible. Those that merit serious consideration are: (*1*) ester bonds between phosphoric acid residues and sugar hydroxyl groups, (*2*) pyrophosphate bonds between two phosphoric acid groups, (*3*) phosphoamide links between phosphoric acid groups and amino groups in the purine or pyrimidine ring, and (*4*) ether links between two carbohydrate residues. It was realized quite early that a distinction may be made between these possibilities by titrating the nucleic acid with acid or base. Electrometric titration of the acid and base groups of nucleic acids has thus become an important method of investigating their structure and other methods, such as dye binding, have also been used to investigate the number and nature of the acid and base groups. Full details of the electrometric titration of nucleic acids will be given in Chapters 9 and 10 and it will suffice here to give only the results of these investigations.

The four possible methods of forming the internucleotide bond given above, if used separately in the formation of nucleic acids, would lead to macromolecules having very different types and numbers of free acid and base groups capable of titration. These are summarized in *Table 10*. It is evident, assuming that only one mode of linkage exists predominantly in the nucleic acid, that it is possible to distinguish between these possibilities by determining the number and nature of the free acid–base groups by electrometric titration.

The earliest studies were carried out by Levene and Simms[1], Jorpes[2] and Makino[3] and similar results were obtained later by Bredereck and co-workers[4,5]. The titration curves obtained all showed the presence of three to four ionizable groups per four atoms of phosphorus on titration to pH 8·0. (Titration results have generally been recorded in this way; the reason stems from the original belief that in all nucleic acids four nucleotides were present in exactly equivalent proportions.) These were regarded by Levene and Simms to be the three amino groups of adenylic, guanylic, and cytidylic acids (see *Table 9*, Chapter 7) and a primary phosphoric acid group. Since the isoelectric point occurred at about pH 1, it was considered that three more primary phosphoric acid groups were also present. On the basis of this evidence (see *Table 10*) it is clear that possibilities (*2*), (*3*), and (*4*) can be rejected and that the phospho-ester linkage is the only possibility. Levene and Simms[6] therefore suggested the following formula (*I*) for ribonucleic acid:



$$
\begin{array}{l}
\mathrm{HO{-}\overset{|}{P}{-}O{-}sugar{-}adenine}\\
\qquad\qquad\quad |\\
\qquad\qquad\quad \mathrm{O}\\
\qquad\qquad\quad |\\
\qquad\qquad \mathrm{HO{-}P{-}O{-}sugar{-}uracil}\\
\qquad\qquad\qquad\qquad\quad |\\
\qquad\qquad\qquad\qquad\quad \mathrm{O}\\
\qquad\qquad\qquad\qquad\quad |\\
\qquad\qquad\qquad\qquad \mathrm{HO{-}P{-}O{-}sugar{-}guanine}\\
\qquad\qquad\qquad\qquad\qquad\qquad\quad |\\
\qquad\qquad\qquad\qquad\qquad\qquad\quad \mathrm{O}\\
\qquad\qquad\qquad\qquad\qquad\qquad\quad |\\
\qquad\qquad\qquad\qquad\qquad\qquad \mathrm{HO{-}P{-}O{-}\underset{|}{sugar}{-}cytosine}
\end{array}
$$

(*I*)

A more careful study of the electrometric titration of yeast ribonucleic acid using refined techniques was made by Allen and

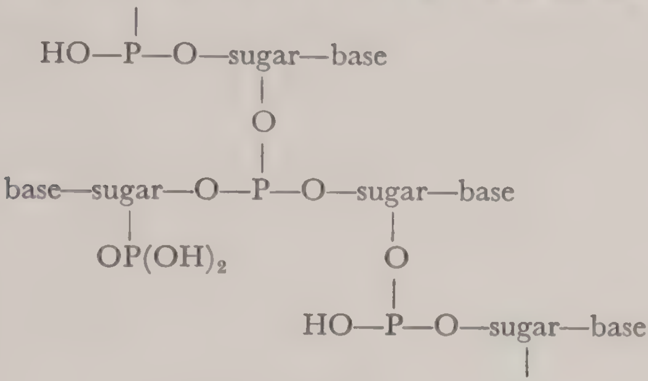
Eiler[7], who were the first to observe that the titration curve indicated the presence of three amino groups for every four atoms of phosphorus, together with one group titrating in the range pH 5·0–7·5. Allen and Eiler considered this last group to be a primary phosphoric acid group that had been considerably weakened by the ionization of the remaining acid dissociations in the macromolecules.

Fletcher, Gulland and Jordan[8] obtained titration results very similar to those of Allen and Eiler but interpreted their results as indicating the presence of three amino groups, one secondary phosphoric acid group, two —NH—CO— dissociations, and three primary phosphoric acid dissociations for every four atoms of phosphorus. This conclusion was confirmed by the titration of deaminated ribonucleic acid in which direct titration of the primary phosphoric acid dissociations is possible. The shape of the titration curve was confirmed by several other workers[9-15], who in general interpreted their results in a similar way although the amount of the secondary phosphoric acid dissociation was found to vary with the different samples of acid used. The presence of only three primary phosphoric acid dissociations and one secondary dissociation was interpreted by Fletcher, Gulland and Jordan[8] as indicating the presence of approximately one triesterified phosphoric acid group for every four atoms of phosphorus, so that in place of the linear polymer of the type envisaged by Levene and Simms (*I*), a branched chain formula (*II*) was suggested. The results, however, still indicated that the internucleotide linkage was a phospho-ester bond. The results of the titration studies and their interpretation were to some extent confirmed by the dye-binding studies of Cavalieri and co-workers[15, 16] and by a kinetic study of the alkaline hydrolysis of ribonucleic acid[17].

Table 10. Titratable groups in polynucleotides with different modes of linkage

Mode of linkage	*Free acid and base groups in polynucleotide*			
	Primary ≡POH	Secondary ≡POH	Amino	—NH—CO—
(*1*) Sugar-phosphate ester	all	none	all	all
(*2*) Pyrophosphate	none	none	all	all
(*3*) Phosphoamide	all	none	none	all
(*4*) Ether	all	all	all	all

The majority of the studies described above were, however, made on degraded samples of ribonucleic acid. That used by Fletcher, Gulland and Jordan[8] was, in fact, a commercial sample almost certainly prepared by the alkaline extraction of yeast. The titration of more carefully prepared and less degraded specimens is therefore of more significance. In recent studies, Crestfield, Smith and Allen[18] found only one secondary phosphoric acid group for every sixteen atoms of phosphorus present in a sample of yeast ribonucleic acid prepared in such a way as to preclude degradation.



(*II*)

A similar result has been observed by Peacocke and co-workers[19, 20] for the carefully prepared samples of the ribonucleic acids of *Aerobacter aerogenes* and yeast. From titration studies it was concluded that there is no detectable secondary phosphoric acid group present in these nucleic acids. It thus appears very probable that the triester group observed by Fletcher, Gulland and Jordan[8] is an artifact produced by degradation of the nucleic acid during preparation.

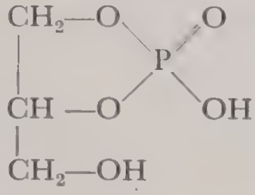
Very strong evidence supporting the absence of any phosphotriester groupings in ribonucleic acids has recently been obtained from the study of the hydrolysis of dialkyl esters of uridine-3′-phosphate by Brown, Magrath and Todd[21]. Alkylation of uridine-3′-phosphate with diazomethane or phenyldiazomethane yields the dimethyl or dibenzyl ester which are, of course, phosphotriesters. These substances were found to be extremely labile and an examination of the stability of the dimethyl ester at 37° C over the pH range 0–9 showed that hydrolysis to the monomethyl ester and a small amount of cyclic phosphate occurred over the whole of this pH range. The half-life of the dimethyl ester was approximately 15 minutes at pH 0 and pH 9, and 75 minutes at pH 7. The extreme lability of these phosphotriesters to even mild hydrolytic conditions is to be compared with the stability of ribonucleic acid under such conditions. Solutions of ribonucleic acid have been maintained at pH 10·6 without any observable liberation of acid groups[22, 23] whereas under these conditions the uridine dialkyl phosphates are readily hydrolysed. If the presence of phosphotriester groupings in ribonucleic acid is still to be regarded as a possibility then it will be necessary to account for their apparent stability in the polynucleotide in contrast to their marked instability in the mononucleotide. Since no abnormal hydrolytic behaviour is observed with the phosphodiester linkages in ribonucleic acids compared with the mononucleotide system, Brown, Magrath and Todd[21] consider that there is no reason to suppose that any phosphotriester groupings would behave in an unexpected manner. The present evidence thus strongly supports the simple linear molecule (*I*), first suggested by Levene and Simms[6].

Having concluded that the internucleotide linkage in ribonucleic acid is a phosphodiester bond formed between the sugar hydroxyl groups of adjacent nucleotides, it is now necessary to determine which hydroxyl groups are involved. Various possibilities arise; the $C'_{(2)}$, $C'_{(3)}$, and $C'_{(5)}$ hydroxyl groups in each nucleoside residue are available for the formation of the internucleotide bond. There are thus six theoretically possible bonds which may be represented diagrammatically (omitting all but the involved oxygen atoms on the phosphorus atoms) as follows:

$C'_{(2)}$—O—P—O—$C'_{(2)}$ $C'_{(3)}$—O—P—O—$C'_{(3)}$ $C'_{(5)}$—O—P—O—$C'_{(5)}$
$C'_{(2)}$—O—P—O—$C'_{(3)}$ $C'_{(3)}$—O—P—O—$C'_{(5)}$
$C'_{(2)}$—O—P—O—$C'_{(5)}$

Some of these obviously could not be the sole mode of linkage in the polynucleotide. Thus a $C'_{(2)}$—O—P—O—$C'_{(2)}$ bond would have to alternate with some other bond not involving a $C'_{(2)}$ position.

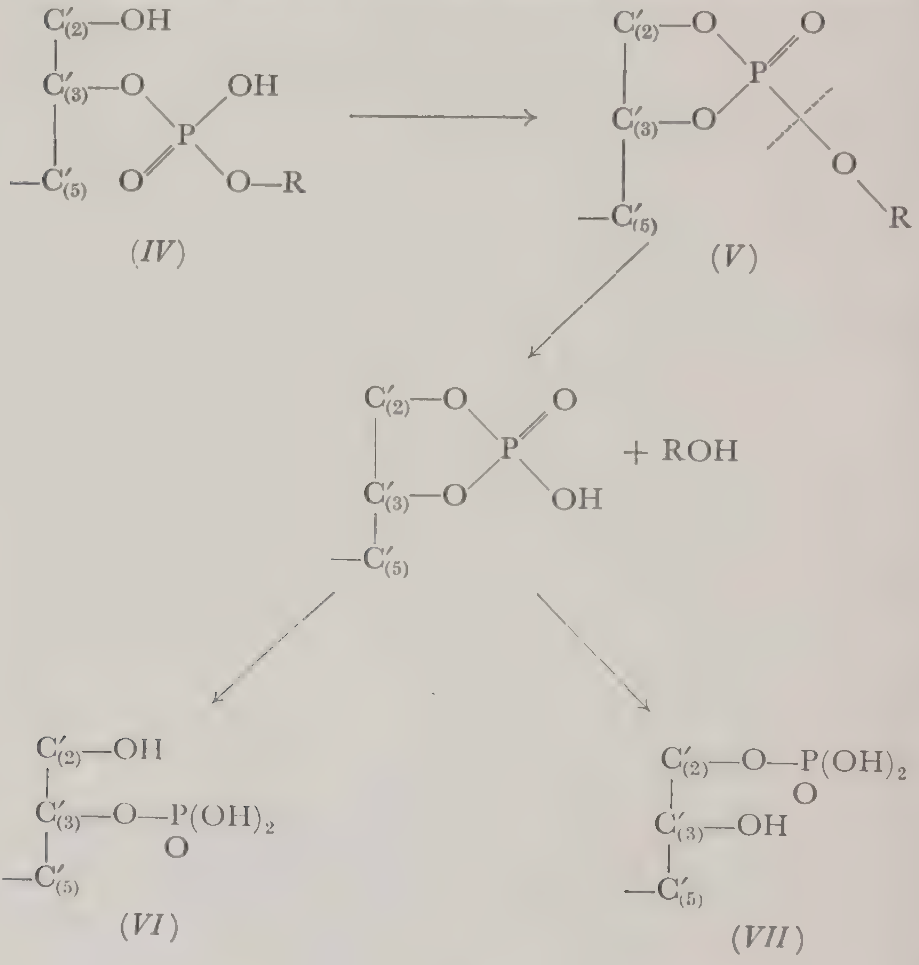
Elimination of some of these possibilities comes from a study of the alkaline hydrolysates of ribonucleic acid. As described in Chapter 6, alkaline hydrolysis of ribonucleic acid yields a mixture of the 2′- and 3′-isomers of all the different nucleotides. Brown and Todd[24] have drawn attention to the similarity of behaviour between the isomeric nucleotides and the glycerol phosphates. Thus the 2′- and 3′-isomers are readily interconvertible under acid conditions, each pure isomer being converted to a mixture containing both isomers, but, on the other hand, the isomers are stable in alkali. The glycerol phosphates behave similarly; glycerol α-phosphate, while being unaffected by alkali, is re-arranged in warm dilute acid solution to an equilibrium mixture of the α- and β-phosphates. This re-arrangement has been shown to be intramolecular by Chargaff[25], who also suggested[25, 26] that the phosphoryl migration occurred through an intermediate cyclic ester (*III*). Furthermore, whereas the α-glycerol phosphate is stable to alkali, methyl-α-glycerol phosphate is readily hydrolysed under alkaline conditions to methyl alcohol and a mixture of the α- and β-glycerol phosphates, it being particularly significant to the argument that no methyl phosphate is produced[27]. Brown and Todd[24] conclude that the pre-requisite for the facile alkaline hydrolysis of a diester of phosphoric acid is the presence of a hydroxyl group in close proximity to the phosphoryl group, the mechanism of the hydrolysis involving the formation of a neutral cyclic phosphotriester. That interaction between neighbouring hydroxyl and phosphoric acid groups takes place had previously been suggested by Kumler and Eiler[28] who showed that the sugar phosphates are abnormally strong acids in comparison with the monoalkyl phosphates. Fonó[29], also, had previously stressed the importance of neighbouring hydroxyl groups in causing lability of the diesters of phosphoric acid containing glycerol or ethylene glycol residues and had also considered the possibility of the formation of a cyclic triester as an intermediate during the hydrolysis. Fonó had, moreover, applied these ideas to the mechanism of the hydrolysis of nucleic acids and had put forward the view that the alkali lability of ribonucleic acid, compared to the stability towards alkali of deoxypentose nucleic acid, could be attributed to the presence of a hydroxyl group at $C'_{(2)}$ in the former.



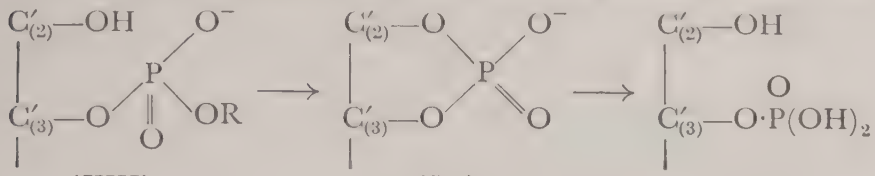
(*III*)

These views were confirmed by Brown and Todd[24] by an examination of the hydrolytic behaviour of the adenosine benzyl hydrogen phosphates. Adenosine-2′- and -3′-benzyl hydrogen phosphates, both of which contain a hydroxyl group adjacent to the phosphoric acid group, are readily hydrolysed by weak alkali at 30°C. Under the same conditions, however, adenosine-5′-benzyl hydrogen phosphate, which contains no hydroxyl group adjacent to the phosphoric acid group, is not affected. Further strong support for this view comes from the preparation of the cyclic esters by Brown, Magrath and Todd[30] and by the identification of the cyclic phosphates in the hydrolysates of ribonucleic acid by Markham and Smith[31].

There is thus very strong support for the view that the first step in the hydrolysis of ribonucleic acids is the formation of a cyclic triester. As has been described above the triesters of phosphoric acid are extremely labile[21], being readily hydrolysed in both acid and alkaline media. The intermediate triester formed during the hydrolysis of ribonucleic acid will thus rapidly hydrolyse. Although in this cyclic intermediate all three ester linkages are of comparable reactivity, only by fission of the bond retaining the singly linked substituent can degradation of the molecule occur. This mechanism is represented diagrammatically by (*IV*)–(*VII*), where the sugar residue is abbreviated to $C'_{(2)}—C'_{(3)}—C'_{(5)}$. Although the mechanism was originally envisaged as involving the neutral triester (*V*) it is now considered that the hydrolytic process depends on the attack

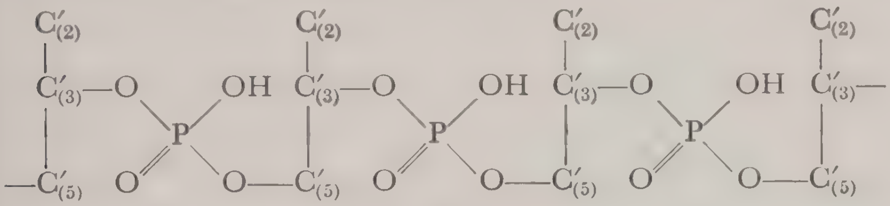


by the vicinal hydroxyl group on the P=O bond, catalysed by either hydrogen or hydroxyl ions, with simultaneous removal of R as an alkoxy ion. The sequence is then represented by (*VIII*)–(*X*)[32]. The enzymic hydrolysis probably proceeds by a similar mechanism.

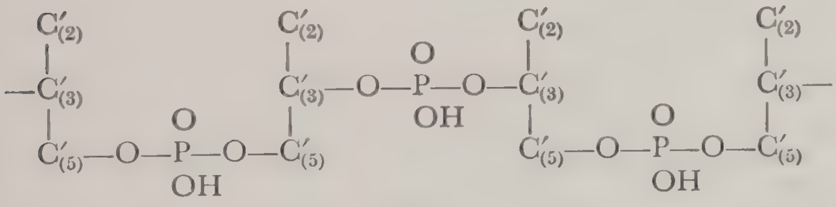


(*VIII*) (*IX*) (*X*)

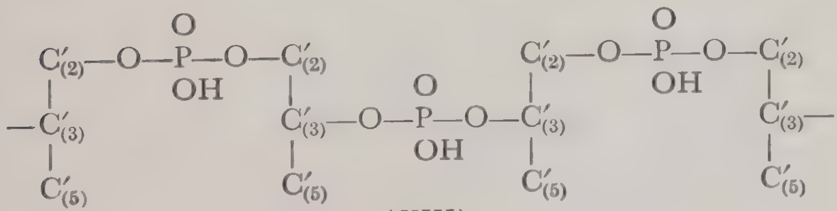
An understanding of the mechanism of the alkaline hydrolysis of ribonucleic acid does not of itself completely define the location of the internucleotide linkages; nevertheless several possible combinations may now be excluded. The establishment of the above mechanism clearly indicates that the 2′- or 3′- positions are involved or possibly both. The possible structures for the internucleotide linkage may thus now be written as (*XI*)–(*XIV*). Alternative structures to (*XI*) and (*XII*) may be formulated in which the $C'_{(2)}$ position is used in place of the $C'_{(3)}$.



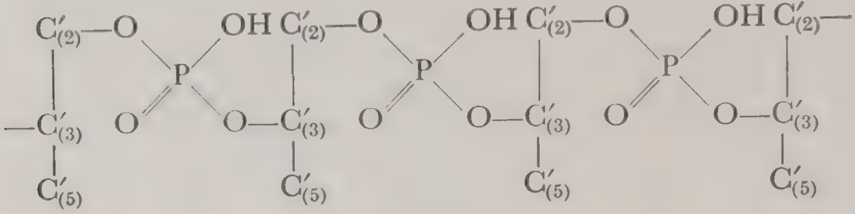
(*XI*)



(*XII*)



(*XIII*)



(*XIV*)

Some of these possible structures may be eliminated by consideration of the nature and products of the hydrolysis[33]. Structure (*XII*), together with the corresponding structure involving the $C'_{(2)}$ position, both of which contain the $C'_{(5)}—C'_{(5)}$ linkage, may be dismissed, since on hydrolysis they would yield dinucleotides stable to further hydrolysis. This view that a $C'_{(5)}—O—P—O—C'_{(5)}$ would be stable to hydrolysis is borne out by the stability of adenosine-5′-benzyl hydrogen phosphate to alkaline hydrolysis referred to above[24] and is confirmed by the observed stability of synthetic dinucleoside-5′,5′-phosphates to alkali. Diuridine-5′, 5′-phosphate[34] (synthesized by Gulland and Smith, and believed erroneously to be the -2′,2′-isomer) and adenosine-5′-uridine-5′-phosphate[35] are both stable to alkali under conditions which bring about complete hydrolysis of ribonucleic acid to mononucleotides. This stability will be due to the absence of one adjacent hydroxyl necessary for cyclization to the triester and subsequent easy fission to mononucleotides.

Structures (*XIII*) and (*XIV*) may be dismissed since they contain no -5′-phosphates and Cohn and Volkin[36, 37] have isolated all four 5′-nucleotides from the hydrolysate produced by the action on ribonucleic acid of snake venom phosphodiesterase, in which the monoesterase activity was inhibited (see Chapter 4).

It thus transpires that structure (*XI*) is the only one which is acceptable on the basis of the experimental evidence and that the internucleotide bond in ribonucleic acid is a phospho-ester linkage joining the $C'_{(2)}$ or $C'_{(3)}$ position on one nucleotide with a $C'_{(5)}$ position on an adjacent nucleotide.

The decision between the $C'_{(2)}$ and $C'_{(3)}$ positions is made difficult, since alkaline hydrolysis proceeds via the cyclic nucleoside-2′, 3′-phosphates and the same mixture of nucleoside-2′- and -3′-phosphates will be obtained regardless of whether a 2′—5′ or 3′—5′ internucleotide linkage occurs in the original ribonucleic acid. However, a solution to this problem has been obtained by the study of the action of nuclease-type enzymes. The action of pancreatic ribonuclease on ribonucleic acid is considered to proceed by a mechanism similar to that of alkaline hydrolysis in that intermediate cyclic phosphates are involved[24, 31, 38, 39]. The action of ribonuclease on uridine-2′- and -3′-benzyl hydrogen phosphate and on cytidine-2′- and -3′-benzyl hydrogen phosphate has been studied by Brown and Todd[32], while the action of enzymic fractions having similar behaviour, from spleen, intestinal mucosa, rye grass and potato on adenosine-2′- and -3′-benzyl hydrogen phosphate and on cytidine-2′- and -3′-benzyl hydrogen phosphate has been investigated by Brown, Heppel and Hilmoe[40]. All the enzymes possess similar behaviour in that they hydrolyse all the -3′-benzyl hydrogen phosphates to the corresponding nucleoside-3′-phosphate, but have no effect on the 2′-isomers. It would thus appear that these enzymes act as diesterases, specifically hydrolysing esters of the nucleoside-3′-phosphates.

The importance of this evidence lies in the action of these enzymes on ribonucleic acid. Ribonuclease does not hydrolyse ribonucleic acid completely to nucleotides, since this enzyme is apparently specific to certain internucleotide bonds, and the products of hydrolysis are pyrimidine nucleotides and oligonucleotides (see Chapter 4). The spleen fraction of Brown, Heppel and Hilmoe[40], however, catalyses the nearly complete hydrolysis of ribonucleic acids, over 90 per cent of the original ribonucleic acid being converted into mononucleotides. Since these enzymes have been shown to be specific for the diester situated at position 3′ on the sugar it necessarily follows that the linkage point in the ribonucleic acids must also be at the 3′- position of the nucleoside residues, regardless of the mechanism of the enzymic hydrolysis. Since it has already been demonstrated that the 5′- position is involved in the internucleotide linkage, it follows that the internucleotide bond in ribonucleic acids is a phosphodiester linking the $C'_{(3)}$- position of one nucleotide with the $C'_{(5)}$- position of the adjacent nucleotide.

This conclusion could be less certain if a cyclic nucleotide was formed as an intermediate during the enzymic hydrolysis. However, Heppel, Whitfield and Markham[42] have shown that the enzymic preparation from spleen, although hydrolysing the nucleoside-2′,3′-phosphates, does so to give only the nucleoside-2′-phosphates. This would indicate that the cyclic intermediates are not formed, and there can be little possibility of migration of the phosphate group during the course of the enzymic hydrolysis. Further support comes from the degradation of dinucleotides resulting from the exhaustive action of ribonuclease on ribonucleic acid. These dinucleotides are composed of a purine nucleoside joined at $C'_{(2)}$ or $C'_{(3)}$ by a phosphodiester bond to the $C'_{(5)}$ of a pyrimidine nucleotide. If the dinucleotide is dephosphorylated by prostate phosphomonoesterase to give a dinucleoside monophosphate, which is then treated with sodium periodate, the residue having free $C'_{(2)}$ and $C'_{(3)}$ (*i.e.* that linked at $C'_{(5)}$ by the phosphodiester group) groups will be oxidized to the dialdehyde. After purification, the product on treatment at pH 10 with Russell viper venom, yielded a derivative of cytosine and a nucleotide identified as a nucleoside-3′-phosphate. It would thus indicate that the point of attachment in the original dinucleotide is $C'_{(3)}$ and not $C'_{(2)}$ to $C'_{(5)}$.

Recently Michelson, Szabo and Todd[43] have synthesized the dinucleotide, adenosine-2′-uridine-5′-phosphate by condensing 3′,5′-diacetyladenosine-2′-benzylphosphorochloridate with 2′,3′-diacetyluridine and subsequently removing the protecting groups. This dinucleotide is not hydrolysed by pancreatic ribonuclease or by the enzymic fraction from spleen, thus confirming the specific action at position $C'_{(3)}$ of these enzymes. Unfortunately the corresponding 3′,5′-dinucleotide, which would be even more relevant to the arguments developed above, has not been prepared.

THE INTERNUCLEOTIDE BOND IN DEOXYPENTOSE NUCLEIC ACIDS

The problem of the internucleotide bond in deoxypentose nucleic acids is simplified in comparison with the related problem in pentose nucleic acid, by the absence of any possibility of $C'_{(2)}$ and $C'_{(3)}$ isomerism of the phosphates owing to the presence of the 2′-deoxy group. The methods which have been used to determine the nature of the internucleotide bond in these acids are very similar to those already described for pentose nucleic acids.

The earliest titration studies on deoxypentose nucleic acids gave conflicting results[1, 3, 44–48], the acid on titration to pH 8·0 being described as containing between four and five acid dissociations for every four atoms of phosphorus. These results were almost certainly due to the presence of a high proportion of secondary phosphoric acid groups in the samples which were almost certainly degraded preparations.

The first study of an acid isolated by a mild procedure was that of Hammarsten[49] who by conductimetric titration observed only four dissociations for every four atoms of phosphorus in this pH range, and the complete absence of any secondary phosphoric acid dissociations. This result was confirmed by the somewhat preliminary titration studies of Jorpes[2] and Stenhagen and Teorell[50].

More extensive studies on carefully prepared, high molecular weight samples of the deoxypentose nucleic acid of calf thymus by Gulland, Jordan and Taylor[51] and later confirmed by Signer and Schwander[52], Cosgrove and Jordan[53] and Lee and Peacocke[54] showed from the reversible back titration curve the presence of 0·3 equivalents, or less, of secondary phosphoric acid dissociation for every four atoms of phosphorus. However, the accurate analysis of the titration curve for secondary phosphoric acid dissociations requires an accurate knowledge of both the pK_a' values and the amounts of the other dissociating groups. The calculations described above assumed that the nucleotides were present in equimolecular proportions, but with the introduction of chromatographic analysis this assumption was found to be quite wrong for the majority of nucleic acids and the appropriate analytical values were used for analysis of the titration curves. The proportion of secondary phosphoric acid groups was then calculated by Lee and Peacocke[54] to be of the order of 0·2–0·4 equivalents for every four atoms of phosphorus. A similar conclusion was reached by Cosgrove and Jordan[55] and by Jordan, Mathieson and Matty[56].

However, this result was still suspect as the chromatographic analyses were not accurate, the recovery of bases being low, and moreover the pK_a' values used were those of the nucleotides and not those of the corresponding groups in the polynucleotide. A further study using more accurate analytical figures and pK_a' values for the acid–base groups obtained from various polynucleotides has been carried out by Cox and Peacocke[57] for a sample of highly polymerized deoxypentose nucleic acid from herring spermatozoa. By this means a theoretical dissociation curve was constructed which fitted the experimental curve closely and which contained no secondary phosphoric acid dissociations. It was thus concluded that if branches or other forms of main chain interruptions occur in deoxypentose nucleic acid they must do so at a frequency too low for detection by electrometric titration, *i.e.* at less than one nucleotide in thirty[58].

It thus appears certain that the internucleotide linkage in deoxypentose nucleic acid, like that in pentose nucleic acid, is a phosphodiester linkage joining a $C'_{(3)}$ atom in one nucleotide to a $C'_{(5)}$ atom in an adjacent nucleotide. The polynucleotide will thus have a similar structure to that already ascribed to pentose nucleic acids (*I*) (p. 141).

Suggestions have been made of other types of linkage. Thus von Euler and Fonó[59] suggested the existence of linkages between phosphate and —NH—CO— groups in order to account for the observed liberation of base-binding groups when deoxypentose nucleic acid is treated with alkali at pH 11·5. A similar suggestion was made by Little and Butler[60] to account for the liberation of groups of pK_a' 9–10 during deoxyribonuclease action. These observations, however, are better explained in terms of internucleotide hydrogen bonds as suggested by Gulland, Jordan and Taylor[51]. Stacey and co-workers[61, 62] and Lee and Peacocke[63], on the basis of the reaction of deoxypentose nucleic acids with Feulgen's reagent after mild treatment with acid or alkali, have postulated the existence of a small number of labile phosphoryl linkages attached to $C_{(1)}$ of deoxyribose residues. However, whether such linkages exist or not, and the evidence for them is not strong, there can be no doubt that the major linkage is the phosphodiester linkage between the sugar residues.

Further evidence supporting the $C'_{(3)}$—$C'_{(5)}$ phosphodiester linkage comes from enzymic and chemical studies. The deoxyribose nucleotides, isolated in almost quantitative yield from the enzymic hydrolysate of deoxyribose nucleic acids using the deoxyribonuclease of calf pancreas, have been shown to be -5′-phosphates by Carter[64] on the basis of reaction with 5′-nucleotidase and by Volkin, Khym and Cohn[65] on the similarity of their chromatographic behaviour with adenosine-5′-phosphate. This evidence confirms the location of one end of the internucleotide linkage on a $C'_{(5)}$ atom. The deoxyribonuclease from calf spleen yields deoxyribonucleotides[66] which have been shown by Koerner and Sinsheimer[67] to be the -3′-phosphates; the deoxyribonuclease from *Micrococcus pyrogenes* also gives the -3′-phosphates[68, 69]. The $C'_{(3)}$ atom is thus also involved in the linkage. The only other possible structure apart from the $C'_{(3)}$—$C'_{(5)}$ linkage is the alternating $C'_{(3)}$—$C'_{(3)}$ and $C'_{(5)}$—$C'_{(5)}$ linkage (*XII*) (p. 147), previously described as a possible structure for pentose nucleic acids and this could not possibly give all the deoxypentose nucleotides, as -5′-phosphates, in quantitative yield on enzymic hydrolysis.

The $C'_{(3)}$—$C'_{(5)}$ internucleotide linkage is still further confirmed by the behaviour towards enzymes of a dithymidine dinucleotide synthesized by Michelson and Todd[70]. The dinucleotide was prepared by condensing 3′-acetylthymidine with thymidine-3′-benzylphosphorochloridate-5′-(dibenzylphosphate) followed by removal of the protecting groups. (This synthesis was, incidentally, the first preparation of a dinucleotide by chemical synthesis.) Such dinucleotides have been separated, but not isolated in substance, from deoxyribonuclease digests of deoxyribose nucleic acids, and their behaviour towards enzymes studied by Markham and Smith[71] and Sinsheimer[72]. The behaviour of the synthetic dinucleotide towards purified rattlesnake venom phosphodiesterase was identical with that of the dinucleotides obtained by enzymic digestion from deoxyribose nucleic acid. Since the linkage is quite definitely $C'_{(3)}$—$C'_{(5)}$ in the synthetic material, that in the natural dinucleotide should also have this configuration. The isolation of thymidine-3′,5-diphosphate and deoxycytidine-3′,5′-diphosphate[73] from the acid hydrolysate of herring spermatozoon deoxypentose nucleic acid also substantiates this mode of linkage.

The inability to bring about the hydrolysis of deoxypentose nucleic acid by alkali is in accordance with the theory advanced by Brown and Todd[24] for the hydrolysis of pentose nucleic acids. The absence of a hydroxyl group on position 2 of the 2-deoxy sugar prevents the formation of the cyclic phosphotriester which is an essential pre-requisite for the facile alkaline hydrolysis of nucleic acids. Deoxypentose nucleic acids are therefore resistant to chemical hydrolysis, which follows the normal course for the hydrolysis of phosphodiesters.

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9

THE STRUCTURE OF NUCLEIC ACIDS: 2 THE WATSON AND CRICK STRUCTURE OF DEOXYRIBOSE NUCLEIC ACID

INTRODUCTION

From the evidence presented in Chapter 8, it was concluded that the deoxypentose nucleic acids are polynucleotides built up from mononucleotides through a 3′—5′ phosphodiester linkage. The study of the macromolecular structure of these nucleic acids, in which x-ray crystallographic studies have played an important role, has led to the conclusion that the deoxyribose nucleic acid molecule consists of two polynucleotide chains, wound together to form a double helix and held together by specific hydrogen bonds. Although the suggestion that specific hydrogen bonds exist between individual polynucleotide chains originally came from titration studies, it will be convenient to discuss the x-ray studies first, not only because of the much greater structural detail obtained therefrom, but also because it is preferable to consider the titration data in relation to a specific molecular model.

X-RAY DIFFRACTION STUDIES

Deoxypentose nucleic acid has generally been studied in the form of its sodium salt which may be isolated in neutral solution by a variety of procedures (see Chapter 2). It is worthy of note that some of the best x-ray diffraction pictures have been obtained using samples prepared by the method of Signer and Schwander. One of the main features of the structure of deoxyribose nucleic acid has been known since 1938 when Astbury and Bell[1] showed that the x-ray fibre photographs gave a prominent reflection which corresponded to a regular spacing of 3·34 Å along the fibre axis. This spacing was interpreted by Astbury and Bell as being due to flat nucleotides packed one on top of the other and standing out perpendicularly to the fibre axis. This rather small internucleotide spacing is in agreement with the somewhat high density of the dried nucleic acid, which is approximately 1·62 g/c.c. Astbury and Bell[1] and later Astbury[2], also concluded from the fibre diagram that the structure pattern of the nucleic acid repeated itself regularly along the axis of the molecule at a distance of about 27 Å. This would correspond to about every eight nucleotides if these were piled one on top of the other. Although Astbury considered that a linear packing of the nucleotides was the most likely arrangement, he did not completely exclude the possibility that the nucleotides might be disposed spiralwise about the long axis of the molecule.

The structural model proposed by Astbury rested, in part, on the assumption that in a nucleoside the purine or pyrimidine ring systems were in the same plane, or at least in a parallel plane, to that containing the sugar ring. This assumption was shown to be incorrect by Furberg[3, 4], who, from an x-ray study, showed that in cytidine the pyrimidine and sugar rings are almost perpendicular. The glycosidic bond, $N_{(3)}$—$C_{(1)}$ having a length of 1·47 Å is clearly a single bond and free rotation about the bond is therefore possible. However, Furberg[5] has shown that, in the crystal, not all orientations of the sugar and pyrimidine rings are equally possible and the most favourable position is that in which the $N_{(3)}\cdot C'_{(1)}\cdot O'_{(1)}$ and $N_{(3)}\cdot C'_{(1)}\cdot C'_{(2)}$ bond angles are 109° and 115° respectively. Since the $C'_{(2)}\cdot C'_{(1)}\cdot O'_{(1)}$ bond angle is 111°, the angle between the two ring systems is almost 90°. The detailed structure of cytidine is shown in *Figure 24* (p. 110). Furberg[3, 4] has also made a preliminary study of the structures of uridine, adenosine and guanosine and has concluded that the general structural features of the nucleosides are very similar. These conclusions on the structure of the nucleosides have been confirmed by x-ray studies of 2′,3′-isopropylidene-3′,5′-cycloadenosine iodide by Clark, Todd and Zussman[6] and by Zussman[7].

Furberg's x-ray studies were confined to the ribonucleosides and it has been generally assumed that in the deoxyribonucleosides the replacement of hydroxyl by hydrogen at $C'_{(2)}$ would be unlikely to cause any very significant structural changes. This view is confirmed by the x-ray study of 5′-bromo-5′-deoxythymidine[8]. Comparison of the bond lengths and angles given in *Figure 26* for this compound with the values in *Figure 25* (pp. 110, 112) for cytidine shows a close similarity.

On the basis of his structure of the nucleosides, Furberg[5] proposed two alternative structures (*Figure 27*) for nucleic acids which were essentially revisions of the structure proposed earlier by Astbury. In these structures the plane of the purine and pyrimidine rings is at right angles to the direction of the long axis of the molecule, but the sugar residues and the P—$O'_{(3)}$ bonds are in planes approximately parallel to the long axis. The majority of the repeating groups, *e.g.* phosphorus atoms, purine and pyrimidine rings *etc.*, are approximately 3·4 Å apart in agreement with the fundamental spacing discovered by Astbury.

Following the successful formulation of the structure of several of the proteins as helical peptide chains, Pauling and Corey[9, 10] suggested a structure for nucleic acid which they considered to be compatible with the main features of the x-ray diagrams obtained by themselves and by Astbury and Bell. They also considered that the structure was in agreement with many of the chemical properties of nucleic acids. The proposed structure involved three intertwined helical polynucleotide chains, each chain having approximately twenty-four nucleotides in seven turns of the helix which has the sense of a right-handed screw. The phosphate groups are closely packed about the long axis of the molecule, with the sugar residues surrounding them and then, on the outside of the helix, the purine and pyrimidine groups project radially, their planes being approximately perpendicular to the molecular axis.

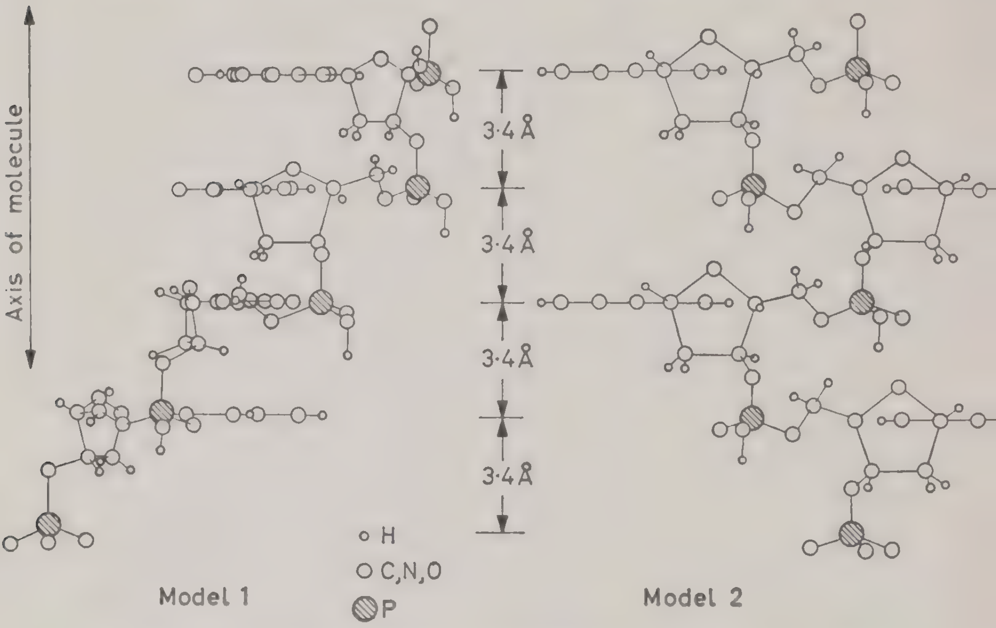

Figure 27. Alternative structures of deoxypentose nucleic acid based on nucleotides of standard configuration (see *Figure 25*, p. 111)
(S. Furberg: *Acta chem. scand.* 1952, **6**, p. 637)

While the Pauling and Corey structure undoubtedly stimulated interest in helical-type structures, there are several points of detail on which it has been criticized. The general behaviour of the nucleate ion in solution suggests that the phosphate groups, instead of being on the inside of the molecule, are freely accessible on the outside of the molecule. The most relevant experimental evidence which supports this alternative configuration is the titration data of Gulland, Jordan and Taylor[11], who demonstrated that the phosphate groups are available for acid–base titration whereas the amino and —NH—CO— dissociations of the purines and pyrimidines are not available for titration in the native nucleic acid, but are bound together by hydrogen bonds. Dye and protein adsorption studies also indicate that the phosphate groups are accessible to large ions. Watson and Crick[12] have also criticized the structure on the basis that many of the van der Waals distances are too short.

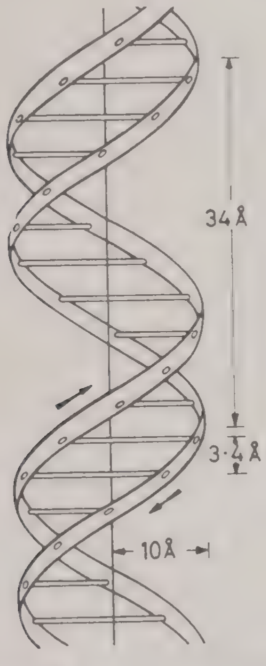

Figure 28. The helical structure of sodium deoxypentose nucleate as proposed by Watson and Crick (J. D. Watson and F. H. C. Crick: *Nature, Lond.* 1953, **171**, p. 737)

In view of these unacceptable features of the Pauling and Corey structure, Watson and Crick[12–14] proposed a new structure for deoxyribose nucleic acid which has many novel features and which appears to be in general agreement with a large amount of different physical and chemical experimental data. This Watson and Crick structure has, except for some alterations to detail, become widely accepted as the basic structure of the deoxyribonucleate ion. The structure consists of two helical polynucleotide chains coiled round the same axis (*Figure 28*), both following right-hand helices but with the sequence of atoms in the sugar-phosphate chain ($C'_{(3)} \cdot C'_{(4)} \cdot C'_{(5)} \cdot O \cdot P \cdot O$) running in opposite directions in the two chains. Each polynucleotide chain in general configuration resembles the model 1 of Furberg (*Figure 27*) and the configuration of the sugar relative to the purines and pyrimidines closely approximates to that in the structure of cytidine also given by Furberg[5] (*Figure 25*). The purines and pyrimidines are thus on the inside of the helix and the phosphate groups on the outside and, in agreement with the x-ray data of Astbury, a nucleotide occurs in the direction of the long axis every 3·4Å. The orientation angle between adjacent nucleotides in the same chain is assumed to be 36° and the structure therefore repeats itself every ten nucleotides or 34Å.

Possibly the most important feature of the structure is the manner in which the two chains are held together by hydrogen bonds between purine and pyrimidines in different chains. The planes of the bases are perpendicular to the molecular axis and two bases, one from each chain, lie in the same plane. Each such pair of bases is held together by hydrogen bonds. This pairing is very specific and only certain pairs of bases can be fitted into the structure. The reason for this specificity lies in the assumption that the sugar-phosphate chain which forms the backbone of each polynucleotide is in the form of a *regular* helix 20Å in diameter. Thus, irrespective of which bases are present, the glycosidic bonds are arranged in a regular manner in space. In particular, any two glycosidic links (one from each chain) which are attached to a hydrogen bonded pair of bases must always occur at a fixed distance apart because of the assumed regularity of the two backbones to which they are joined. The result of this requirement is that one member of a hydrogen bonded pair must always be a purine and the other a pyrimidine in order to form a bridge between the two chains. If a pair consisted of two purines, there would not be enough room between the chains for it; if of two pyrimidines they would be too far apart to form hydrogen bonds.

Each base can exist in a number of tautomeric forms, differing in the exact positions at which the hydrogen atoms are located. However, under physiological conditions, one particular form of each base is much more probable than any other. If it be assumed that these most probable forms always occur for the various bases, then the pairing requirements become even more restrictive and it is found that the only possible pairs of bases are:

adenine with thymine
guanine with cytosine.

According to Watson and Crick[13], 5-methylcytosine or 5-hydroxymethylcytosine may replace cytosine in this structure. The pairing of these bases is shown in *Figures 29* and *30*. If adenine is paired with cytosine, it would not form hydrogen bonds since there would be two hydrogens near one bonding position and none at the other. Similarly if guanine is bonded with thymine, there would be two hydrogens near both bonding positions.

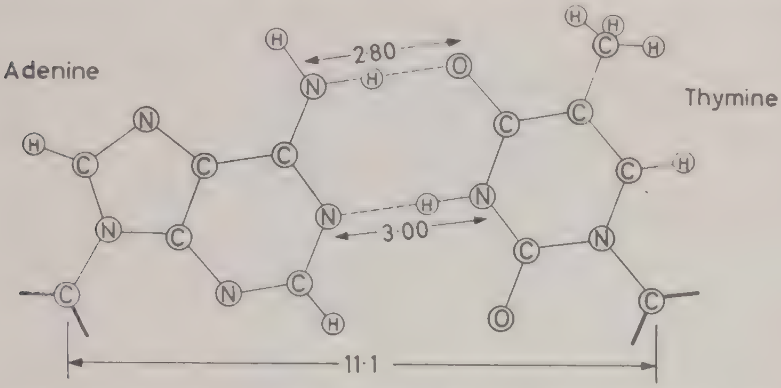

Figure 29. Specific hydrogen bonding in the base pair adenine and thymine

(J. D. Watson and F. H. C. Crick: *Cold Spr. Harb. Symp. quant. Biol.* 1953, **18**, p. 126)

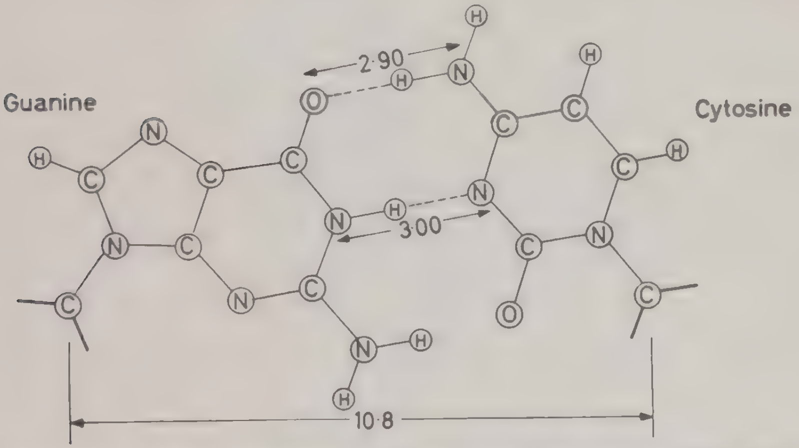

Figure 30. Specific hydrogen bonding in the base pair guanine and cytosine

(J. D. Watson and F. H. C. Crick: *Cold Spr. Harb. Symp. quant. Biol.* 1953, **18**, p. 126)

A given pair of bases may be either way round. For example, adenine can occur in either chain, but when it does its partner on the other chain must always be thymine. However, the structure places no restriction on the sequence of pairs; whatever pair of bases occurs at one particular point in the structure, there is no restriction on the nature of the neighbouring pairs. This is because all the bases are flat and are stacked one above the other. Although there is no restriction of the sequence, the specific pairing demands a definite relationship between the sequences in the two polynucleotide chains; thus each chain must be the complement of the other.

This structure, although based to some extent on the x-ray diffraction data of Astbury and Bell[1] and of Pauling and Corey[9, 10], is largely a hypothetical structure conceived by the actual process of building the model so that it conformed with the known stereochemical data. Confirmation of the general features of the structure, together with precise details concerning the dimensions and some important modifications of the original structure, have been supplied by the x-ray data of Wilkins, Stokes and Wilson[15], of Franklin and Gosling[16–19], of Feughelman, Langridge, Seeds, Stokes, Wilson, Hooper, Wilkins, Barclay and Hamilton[20], and of Langridge, Seeds, Wilson, Hooper, Wilkins and Hamilton[20a] which have also been described by Wilkins[21]. Fibrous crystals of the sodium salt of deoxypentose nucleic acid may be prepared[18] with a high degree of orientation, by withdrawing a needle point slowly from a stiff gel of the nucleate. Varying the speed of withdrawal and the water content of the gel produces fibres of diameters between 1 and 100 mμ. Such fibres give two distinct types of x-ray diffraction pattern, depending upon the water content of the fibre which may be changed by altering the relative humidity of the atmosphere surrounding the crystal. At 75 per cent relative humidity, which corresponds to a water content of the fibre of 40–45 per cent of the dry weight, a high degree of crystallinity exists in the fibre as shown by the large number of sharp spots on the x-ray diagram (*Plate 1*). This form of the nucleic acid has been termed the A configuration[17]. At higher humidities (92 per cent) a different structure (B configuration), which is often paracrystalline, is obtained. The x-ray diagram of this form (*Plate 2*) as the sodium salt, shows few sharp spots which is indicative of imperfect crystallinity. This B configuration appears to persist over a wide range of humidity; the change from the A to the B forms is, however, normally reversible.

Analysis of the x-ray diagrams of the sodium deoxypentose nucleate from *Escherichia coli*[15] and of the sodium deoxyribose nucleate of calf thymus[16] leads to a general confirmation of the main features

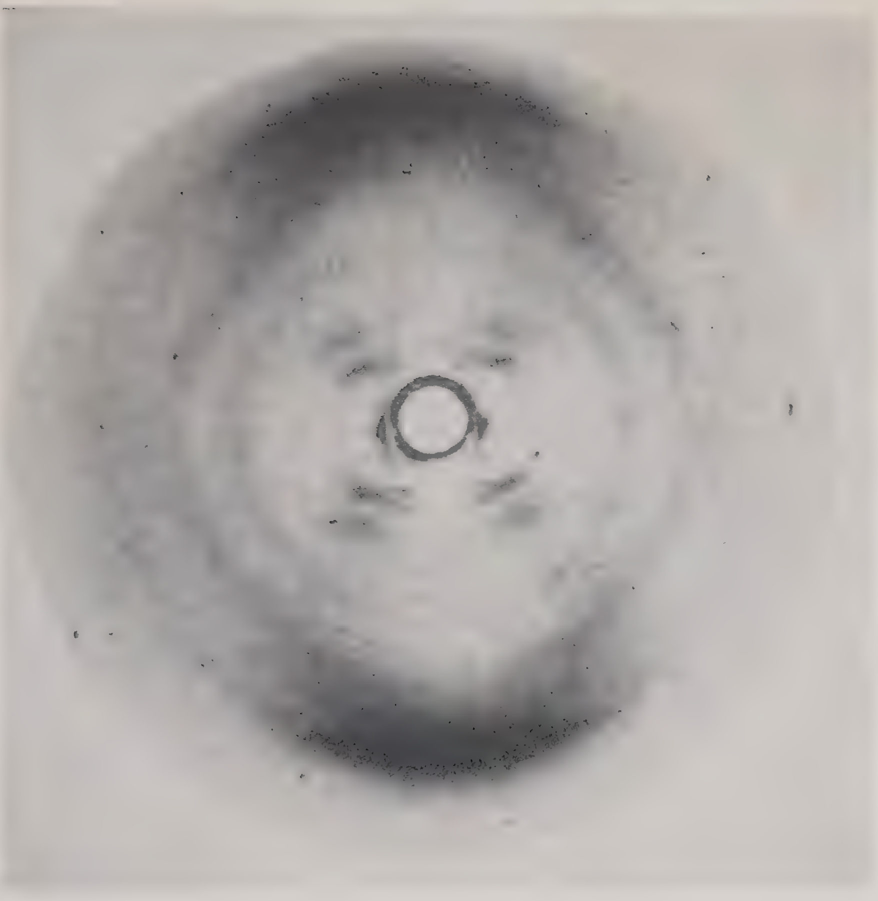

Plate 1. X-ray diffraction diagram of the crystalline A form of sodium deoxypentose nucleate

(R. E. Franklin and R. G. Gosling: *Acta Cryst.* 1953, **6**, p. 674)

(*To face page 162*)

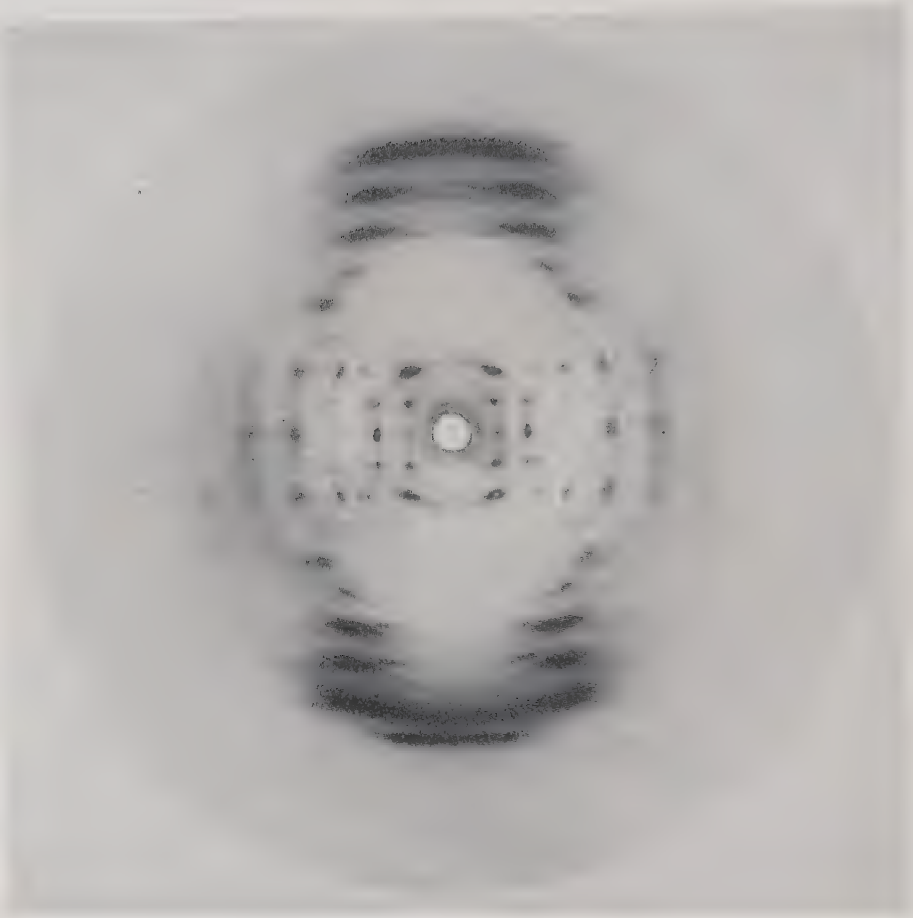

Plate 2. X-ray diffraction diagram of the paracrystalline B form of sodium deoxypentose nucleate
(R. E. Franklin and R. G. Gosling: *Acta Cryst*. 1953, **6,** p. 674)

(To face page 163)

of the Watson and Crick structure. More detailed analysis by Franklin and Gosling[17, 19] of the x-ray diagram of the A form by calculating the cylindrical Patterson function gave direct evidence that the structure is composed of two helical chains. The theoretical curves of a Patterson function for a smooth two chain helix of radius 9·0 Å and in which the two chains are separated by one-half the length of the *c* axis of the unit cell, are found to pass through a large proportion of the Patterson peaks (*Figure 31*). The complete three-dimensional Patterson function suggests that only a part of the structure repeats itself in the plane at $c = \frac{1}{2}$ in the unit cell. This is the result to be expected for two co-axial chains related by a dyad axis, as suggested by Watson and Crick, the third co-axial chain being absent in nucleic acid.

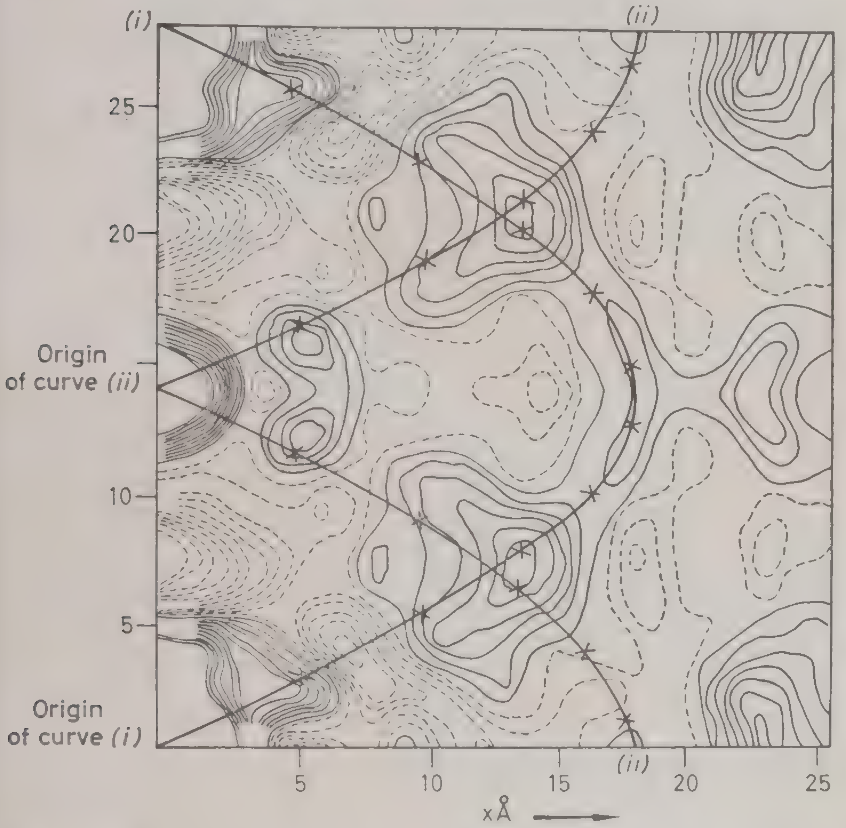

Figure 31. Cylindrical Patterson function for crystalline form of sodium pentose nucleate

(R. E. Franklin and R. G. Gosling: *Nature, Lond.* 1953, **171**, p. 156)

More refined x-ray studies by Wilkins and collaborators[20, 20a, 21] have shown that, while the general features of the Watson and Crick model are correct, some modifications are necessary as the calculated Fourier transform of the Watson–Crick model did not agree with the observed x-ray pattern. The main feature which required correction was the diameter of the molecule. This had been put (somewhat arbitrarily) by Watson and Crick at 20 Å, which was too large. Furthermore, the shape of the hydrogen bonded base pair has been shown[15, 20] to be roughly that of a rod passing approximately through the helix axis. This feature can be achieved if the base pair in the Watson and Crick structure is moved towards the helix axis, a process which also achieves the desired effect of reducing the molecular diameter. Furthermore, the distribution of electron density in the molecule must not alter suddenly at a particular radius of the helix, but decrease gradually near the outside of the molecule, thus reducing the magnitude of secondary diffraction maxima on the equator of the diffraction pattern. This may be achieved if the sugar ring in each nucleotide is placed so that its plane is inclined as much as possible to the axis of the helix.

If molecular models of deoxyribose nucleic acid are built in conformation with the above ideas and with the sugar ring non-planar as in ribose, many interatomic distances are found to be always much smaller than the usually accepted van der Waals values. However, if the models are built on the assumption that the sugar ring is planar, which is not unreasonable in view of the many forces of electrostatic origin present in the vicinity of the nucleate ion, these van der Waals distances become more normal[20].

Further improvements in technique have come from the use of lithium and rubidium salts of deoxyribose nucleic acid in place of the sodium salts[20a]. The lithium salts crystallize in the B configuration and the x-ray photograph consists of sharply defined spots (*Plate 3*) which may be measured accurately. Another advantage of the lithium salt is that the lithium ion scatters about three times less x-rays than the sodium ion and thus may be ignored in the calculation of the diffracted intensities from the molecular model. A molecular model built using accepted stereochemical data, together with those given by Dunitz and Rollett[22] for dibenzylphosphoric acid, and by Huber[8] for 5′-bromo-5′-deoxythymidine, gives a satisfactory correspondence between diffraction intensities calculated from the model and measured experimentally[21] (*Figure 32a*). The disposition of a base pair, in this model, in relation to the helix and diad axes is given in *Figure 32b*. It will be seen that in this model the helix axis passes through the hydrogen

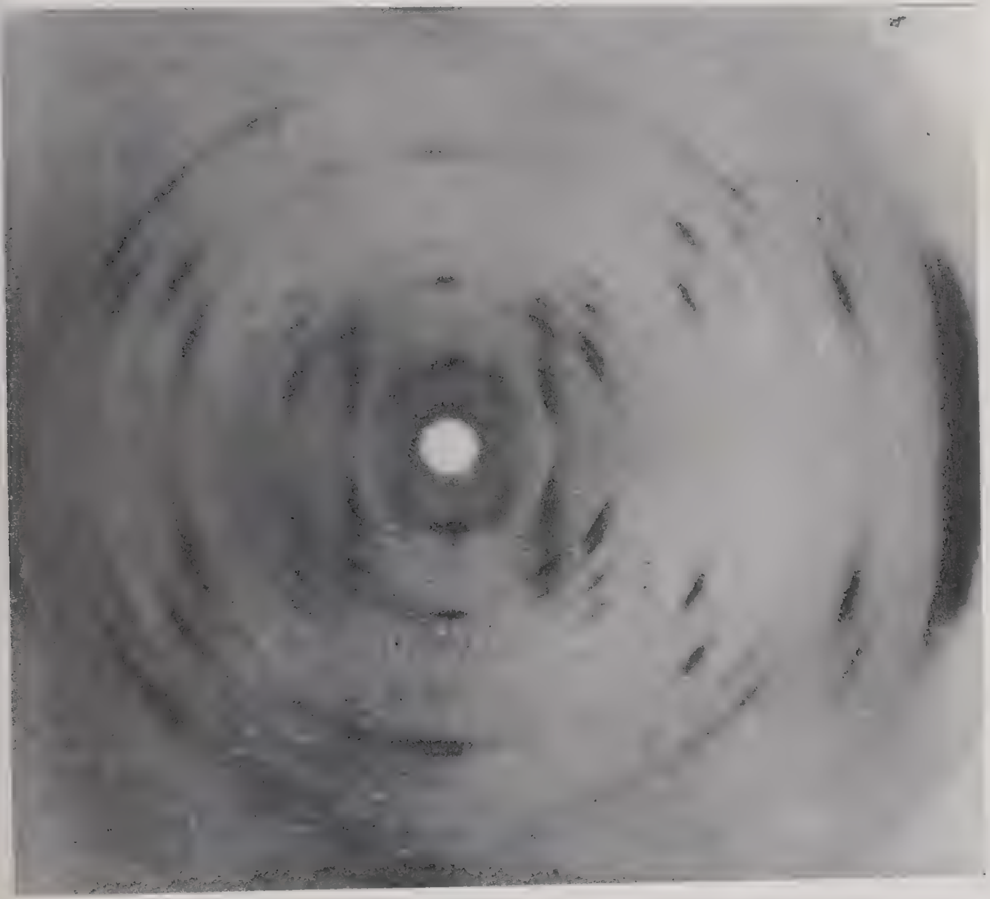

Plate 3. X-ray diffraction diagram of B configuration of lithium deoxypentose nucleate

(M. H. F. Wilkins: *Cold. Spr. Harb. Symp. quant. Biol.* 1956, **21,** p. 76)

(To face page 164)

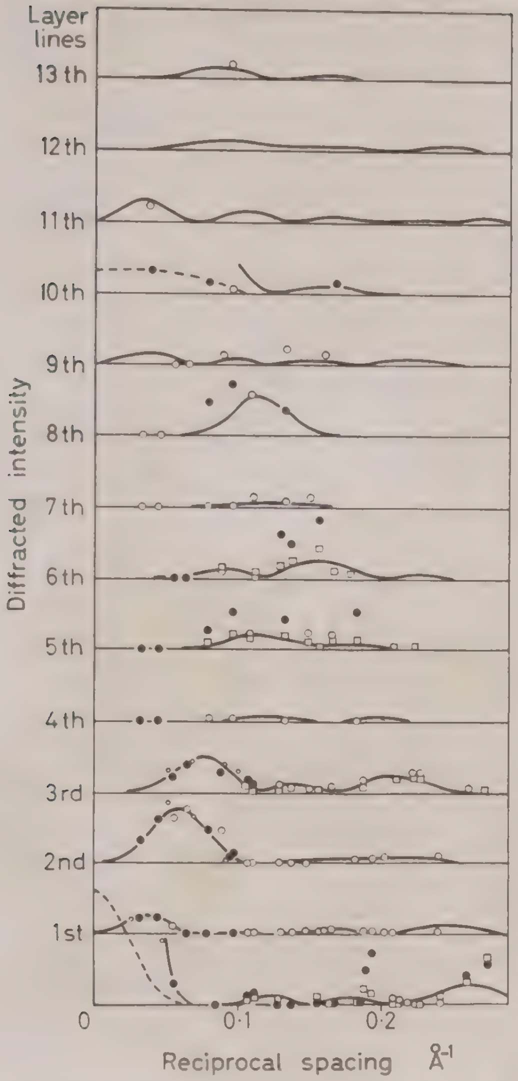

Figure 32a. Agreement between the observed and calculated intensities of diffraction for lithium DNA in the B configuration. The more reliable observed values are shown by filled black circles and less reliable values by unfilled circles of the same size. The small unfilled circles show values for sodium DNA. The smooth curve is the sum of the squares of the calculated amplitudes, and where these terms interfere noticeably, the intensities have been calculated individually for each x-ray spot and are shown by square symbols. Intensities on the 10th layer line and the dotted curve on the zero layer line are plotted on 1/10 scale.

(R. Langridge, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins and L. D. Hamilton: *J. biophys. biochem. Cytol.* 1957, **3**, p. 774) bonded ring system in contrast to the position originally suggested by Watson and Crick (A in *Figure 32b*). A diagrammatic representation of the molecule is shown in *Figure 33* and the differences in configuration of the A and B forms shown diagrammatically in *Figure 34*. This model is strongly supported by the infra-red spectra studies of Sutherland and Tsuboi[23].

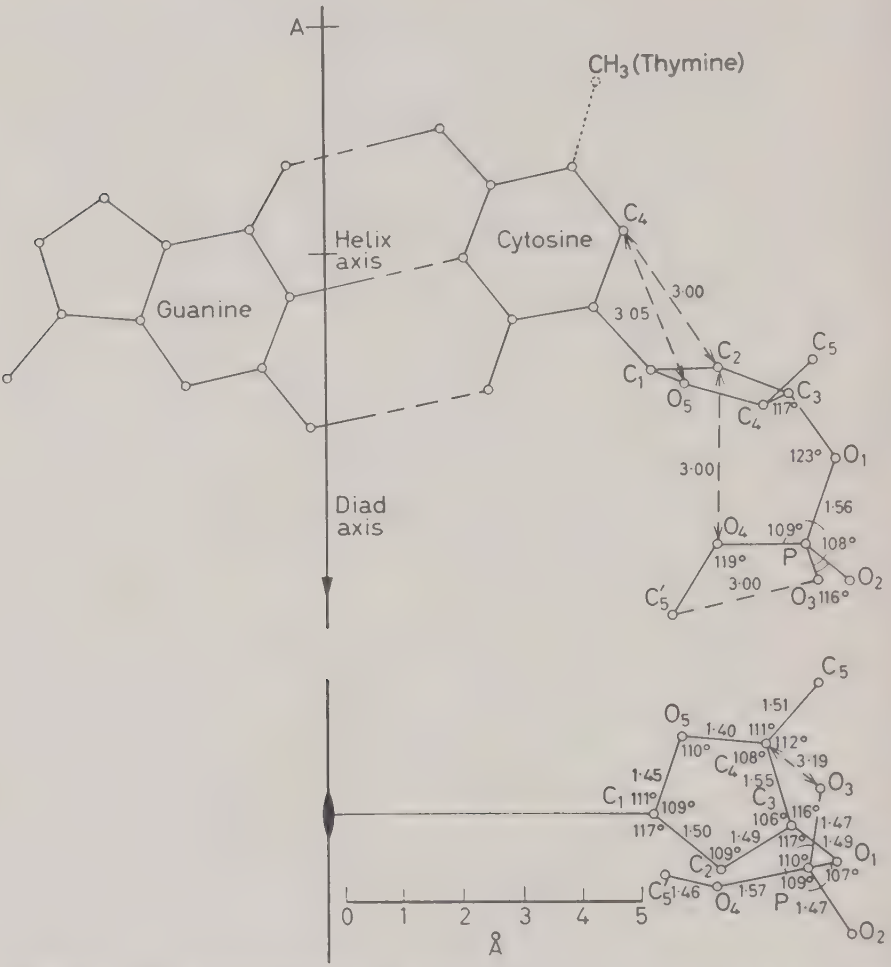
A
CH_3 (Thymine)
Helix axis
Cytosine
Guanine
Diad axis
C_4
3·00
3 05
C_2
C_5
C_1
O_5
C_3
C_4
117°
123°
O_1
3·00
1·56
O_4
109°
108°
119°
P
3·00
O_2
C_5'
O_3
116°
C_5
1·51
O_5
1·40
111°
112°
110°
108°
C_4
3·19
1·45
1·55
O_3
C_1
111°
109°
C_3
106°
116°
1·47
117°
1·50
1·49
117°
1·49
109°
110°
O_1
C_2
107°
C_5
1·46
1·57
109°
O_4
P
1·47
O_2
0
1
2
3
4
5
Å

Figure 32b. The B configuration of deoxyribonucleic acid showing a base pair and projections of a nucleotide on planes perpendicular to the helix axis and to the diad axis

The position of the helix axis of the Watson–Crick model is shown at A (R. Langridge, W. E. Seeds, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins and L. D. Hamilton: *J. biophys. biochem. Cytol.* 1957, **3**, p. 767)

The general dimensions and characteristics of the two forms may be summarized as follows. In the crystalline A form, the two polynucleotide chains form helices, 18 Å in diameter, spaced 14 Å apart along the axis of the helix. The pitch of the helix is 28·1 Å and there are eleven nucleotides in each chain per complete turn. The pairs of bases form a rod, inclined at an angle of about 65° to the axis of the helix, but not passing through the axis. In the B form the pitch of the helix increases to 33·1 Å and the number of

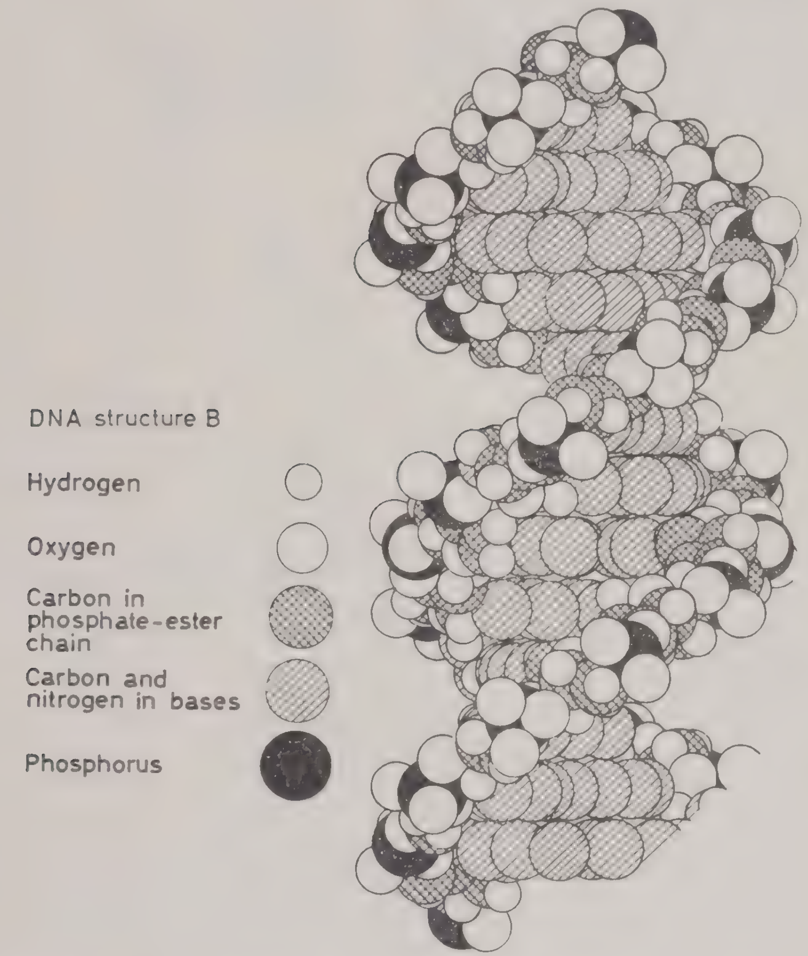
DNA structure B
Hydrogen
Oxygen
Carbon in phosphate-ester chain
Carbon and nitrogen in bases
Phosphorus

Figure 33. The proposed structure of sodium deoxyribose nucleate B (M. Feughelman, R. Langridge, W. E. Seeds, A. R. Stokes, H. R. Wilson, C. W. Hooper, M. F. H. Wilkins, R. K. Barclay and L. D. Hamilton: *Nature, Lond.* 1955, **175**, p. 834)

nucleotides in each turn reduces to ten. The pairs of bases form rods perpendicular to, and passing through, the axis of the helix and the diameter of the helix is slightly reduced (*Figure 34*).

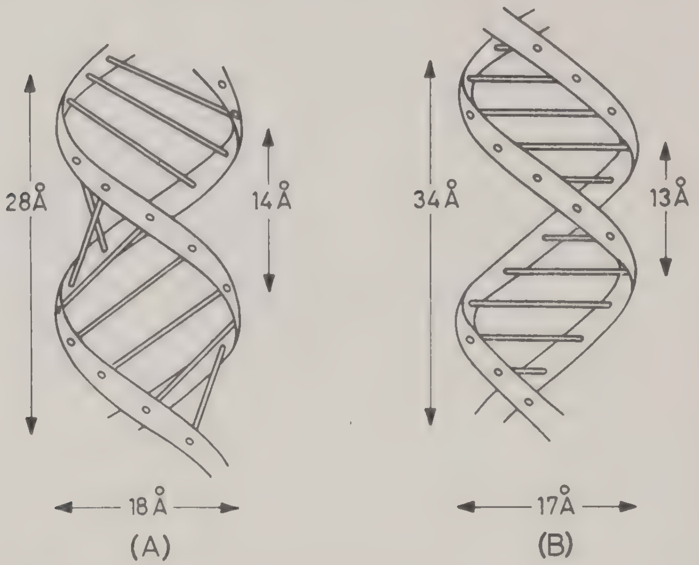
28Å
14Å
18Å
(A)
34Å
13Å
17Å
(B)

Figure 34. Diagram to show the main differences between the proposed models of the A and B forms of sodium deoxypentose nucleate

(G. B. B. M. Sutherland and M. Tsuboi : *Proc. Roy. Soc. A* 1957, **239,** p. 459)

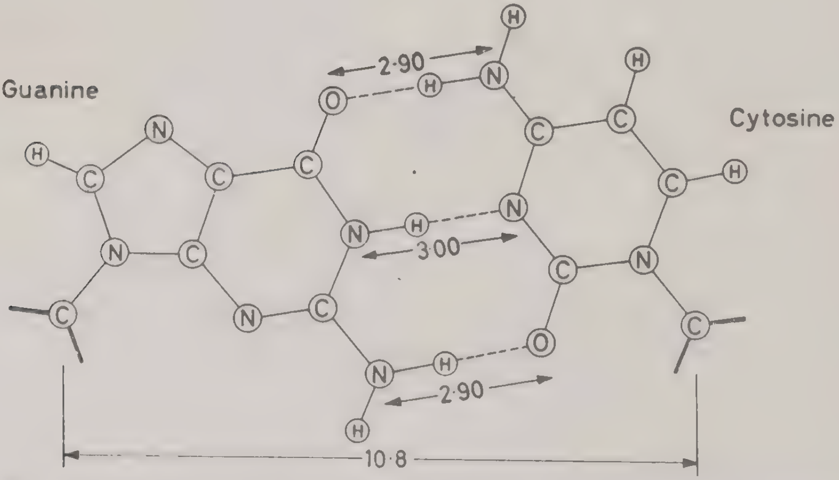
Guanine
Cytosine
2·90
3·00
2·90
10·8
H
N
C
O

Figure 35. Specific hydrogen bonding in the base pair guanine and cytosine according to Pauling and Corey[24]

Recently Pauling and Corey[24] have pointed to the possibility that a third hydrogen bond could exist between cytosine and guanine (*Figure 35*) involving the amino hydrogen of guanine and the keto oxygen of cytosine. The presence of this third hydrogen bond would make the guanine–cytosine base pair more stable than that of adenine–thymine. This has been confirmed experimentally by the heat denaturation of deoxypentose nucleic acids of different composition, those with high guanine and cytosine composition possessing the higher denaturation temperatures. (See Chapter 11, *Figure 76*, p. 262.)

ELECTROMETRIC TITRATION STUDIES

In an electrometric titration study aimed primarily at determining the nature of the groups taking part in the internucleotide bond in deoxyribose nucleic acid of calf thymus, Gulland, Jordan and Taylor[11] observed that the titration curves obtained when solutions of the sodium salt of deoxyribose nucleic acid, originally at pH 6–7, are first titrated to pH 2·5 or to pH 12, are irreversible and differ markedly from the titration curves obtained on back-titration with alkali and acid respectively. This phenomenon is represented in *Figure 36*. It is evident that the addition of acid or alkali to the deoxyribonucleate does not at first bring about the ionization of the acid and base groups and the unbuffered region which exists from pH 6–9 in the back-titration curve extends from pH 4·5–11·0 for the initial titration curve. Outside these limits there occurs a rapid liberation of acid–base groups titrating in the range pH 2·0–6·0 and pH 9·0–12·0. According to Gulland, Jordan and Taylor[11], the same back-titration curve is obtained irrespective of whether the titration is commenced at pH 12 or 2·5. This titration behaviour of carefully prepared specimens of deoxypentose nucleic acid has been confirmed for deoxypentose nucleic acids from other sources and for different preparation procedures by Signer and Schwander[25], Cosgrove and Jordan[26], and Lee and Peacocke[27]. It has also been confirmed by spectrophotometric titration by Shack and Thompsett[28].

The interpretation of this titration phenomenon given by Gulland, Jordan and Taylor was that the amino groups of the purines and pyrimidines were linked by hydrogen bonds to the —NH—CO— groups. The titration of one group would thus bring about the liberation of both groups in accordance with the experimental data. That the bonds were intermolecular and not intramolecular was determined by the viscosity data of Creeth, Gulland and Jordan[29] who observed a very marked decrease in the viscosity of solutions of deoxyribonucleate at those pH values where the ionization of groups occurred. This fall in viscosity was also accompanied by the disappearance of streaming birefringence[29-31] (*Figures 37* and *38*). This could only be explained by a breakdown of the hydrogen bonded structure to yield either smaller molecular units or a less asymmetric macromolecule.

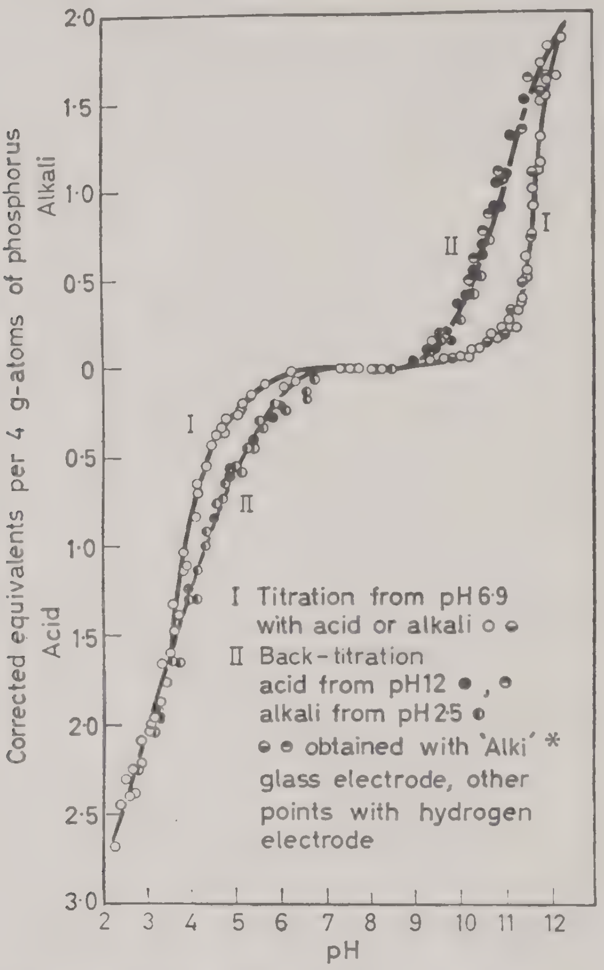

Figure 36. The titration curve of sodium deoxyribose nucleate of calf thymus

The smooth curve drawn through the points in I is calculated for 1 equiv. each of pK_a' values 2·6, 3·5, 5·2, 10·4 and 11·4

* Cambridge Instrument Company, Limited

(J. M. Gulland, D. O. Jordan and H. F. W. Taylor: *J. chem. Soc.* 1947, p. 1132)

These results can now be completely interpreted on the basis of the model of Watson and Crick. The hydrogen bonds joining the specific pairs of nucleotides (*Figures 29* and *35*) involve the amino groups of adenine, guanine and cytosine and the —NH—CO— groups of guanine and thymine. The stability of the hydrogen bond ring system weakens the acidic and basic strengths of the participating groups, thus explaining the irreversibility of the initial titration curve.

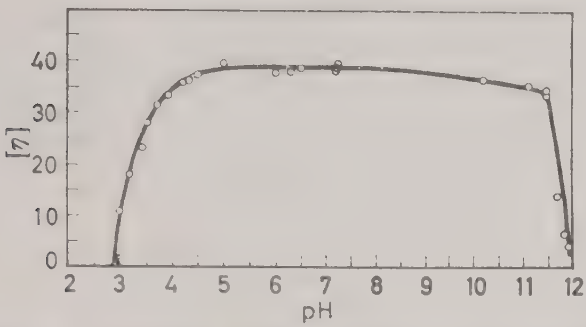

Figure 37. Variation of intrinsic viscosity with pH for sodium deoxyribose nucleate of calf thymus
(From A. R. Mathieson and S. Matty: *J. Polym. Sci.* 1957, **23**, p. 759)

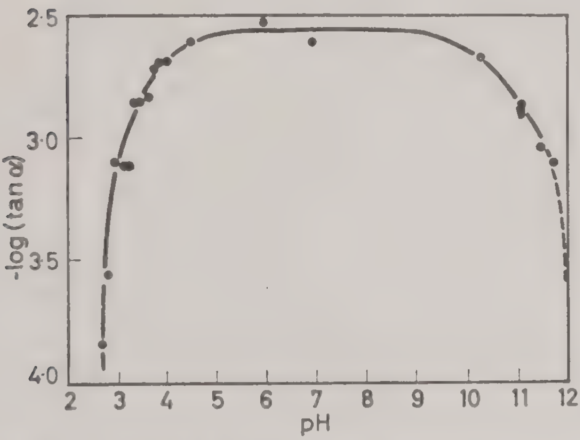

Figure 38. Variation of streaming birefringence with pH for sodium deoxyribose nucleate of calf thymus
(A. R. Mathieson and S. Matty: *J. Polym. Sci.* 1957, **23**, p. 758)

Since the first discovery of this phenomenon the use of refined titration techniques has been directed towards the effect of ionic strength and concentration of nucleate on the titration curve, to the problem of the coincidence of the back-titration curves from pH 2·0 and 12·0 and to the determination of the dissociation constants of the dissociating groups of the polynucleotides.

Jordan, Mathieson and Matty[32] and Cox and Peacocke[33] have shown that the irreversible titration phenomenon (sometimes referred to, somewhat incorrectly, as a hysteresis) is observed over a wide range of ionic strengths and nucleate concentrations. This implies that the phenomenon is not due to interactions between molecules but to true intramolecular (or interpolynucleotide) bonds in accordance with the double-helical model.

Use of the purine and pyrimidine composition of the deoxyribonucleate obtained from chromatographic analyses has enabled the absence of any detectable secondary phosphoric acid dissociation to be established. It has also enabled the titration constants (pG′ values) of the amino groups at 25°C to be determined[34]. These are:

	Guanine	*Adenine*	*Cytosine*
pG′, forward titration	2·50	4·40	4·40
pG′, backward titration	2·40	3·65	4·85

It will be observed that adenine and cytosine possess the same pG′ value on the forward titration, but widely different values on backward titration. Examination of the hydrogen bonded ring systems of the Watson and Crick model (*Figures 29* and *35*) shows that the amino groups of adenine and cytosine are members of very similar eight membered rings. These have to be broken by titration. It is not surprising therefore that the pG′ values are different for these groups in the hydrogen bonded and non-hydrogen bonded states and moreover that their pG′ values are the same in the hydrogen bonded form. The similarity of the pG′ values of guanine for the forward and backward titration curves has sometimes been attributed to the non-participation of the guanine amino group in hydrogen bonding between the bases. However, Cavalieri and Rosenberg[35] have shown that at 1°C the difference between the forward and backward titration curves continues throughout the titration of the guanine amino group (see Chapter 11, *Figure 70*, p. 255) thus confirming that this group also participates in the hydrogen bond formation. The variation of the titration curve with temperature is to be attributed in part to the interdependence of acid and heat denaturation (see p. 254).

Although the original titration curves of Gulland, Jordan and

Taylor showed exact coincidence of the backward titration curves, this has not always been confirmed by more recent studies[27, 33]. This is of structural significance according to Peacocke[36] since, if the two back-titration curves are *exactly* coincident, this would exclude various suggested labile linkages on the amino and —NH—CO— groups (see Chapter 8, p. 151) and confirm hydrogen bonds between them as the only possible means of linkage.

The dissociation curves of herring spermatozoon sodium deoxypentose nucleate have been examined by Cox and Peacocke[33] by a non-continuous method in which a cell without liquid junction is used and which permits precise control of ionic strength. (The more usual method of titration is to use a glass or hydrogen electrode and to add alkali or acid continuously; the ionic strength of the solution is thus not constant during the titration.) A set of results is shown in *Figure 39*, where each point represents the results of several determinations of individual mixtures. The points obtained on back-titration, after ionization of the amino or —NH—CO— groups at pH 2 or 12 respectively, lie on exactly the same curve

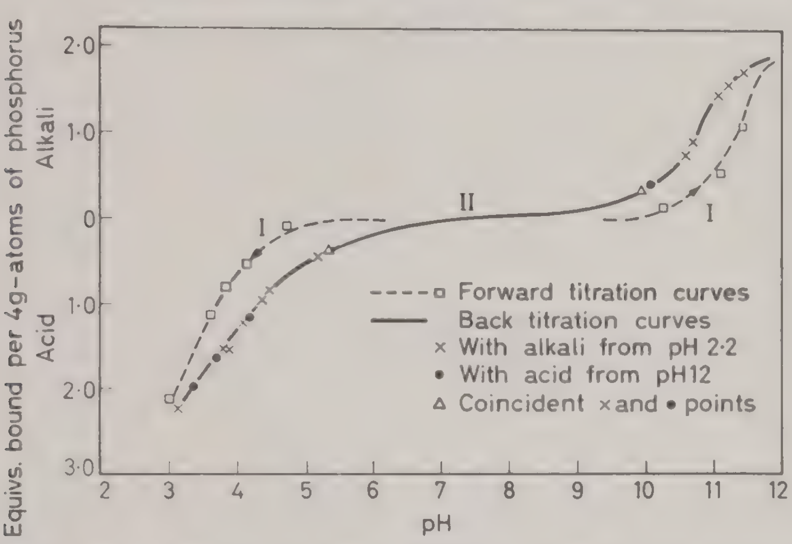

Figure 39. The titration of sodium deoxyribose nucleate from herring spermatozoa obtained on individual mixtures in a cell without liquid junction (25°C, ionic strength 0·05)

I, Forward titration curves from pH 6 to 7 with acid to pH 2·2 or alkali to pH 12

II, Back-titration curves

(A. R. Peacocke: Special Publication of the Chemical Society No. 8, London, 1957, p. 151) which is completely reversible. Thus Peacocke[36] concludes that the amino groups must be joined to the —NH—CO— groups by hydrogen bonds which are broken on ionization. This would indicate that no significant number of these groups, detectable by titration methods, are bonded in any other way. The differences previously observed between the back-titration curves must have arisen through factors associated with the continuous method of titration.

ANALYTICAL EVIDENCE FOR THE WATSON AND CRICK MODEL

The specific pairing of bases in the hydrogen bonded double helix carries with it the implication that for every guanine residue present in the nucleic acid molecule, there must be an exactly equivalent number of cytosine residues. Similarly, adenine and thymine must be present in equivalent amounts. The analytical figures for a large number of deoxypentose nucleic acids show that this is approximately true even though, in nucleic acids from different samples, the ratio of adenine to guanine can vary very considerably. This is illustrated by *Table 6* (Chapter 4, p. 89) which in columns 7 and 8 gives the ratios of adenine to thymine and of guanine to cytosine (plus 5-methylcytosine) respectively. In hardly any case (except *pneumococcus* type III) is there a significant deviation from the figure of unity predicted by the Watson and Crick model, even though the ratio of adenine and thymine to guanine and cytosine (column 9) which is virtually the adenine to guanine ratio, varies over quite wide limits. Watson and Crick[14] consider that these analytical data offer most important experimental evidence in support of their model. However, the very careful analytical figures of the various fractions of nucleic acid obtained by Bendich and co-workers and given in *Table 1* (Chapter 3, p. 53) show that the adenine to thymine and guanine to cytosine ratios are significantly different from unity in some of the fractions. This must indicate that in some fractions the molecules contain more adenine residues than can be hydrogen bonded to thymine, a fact that may have genetic significance, and that the over-all ratio of unity obtained on a total sample may be fortuitous. Until a systematic study by titration and other methods can be made on individual fractions, the exact significance of this observation cannot be assessed. Nevertheless, in view of this new evidence, the analytical data cannot be regarded as giving the support to the Watson–Crick model that was once claimed[14].

POSSIBLE MODIFICATIONS OF THE WATSON AND CRICK STRUCTURE

The Watson and Crick structure assumes that the single deoxypentose nucleic acid molecule consists of two polynucleotide chains, continuous throughout their whole length and bonded together by specific hydrogen bonds. If the polynucleotide chains were not continuous, but had random or regular breaks along their length, the x-ray picture would not be affected so long as the breaks did not occur at opposite positions in the two chains when, obviously, the molecule would break into two. A rather tentative suggestion that a discontinuous structure might occur in the deoxypentose nucleic acid molecule was made by Jordan[37], but a more definite structure, based on experimental evidence and on the Watson–Crick model, has been produced by Dekker and Schachman[38]. The evidence for this model arises largely from studies of the size and shape of the nucleate ion in solution and in the effect of agents which bring about the rupture of the hydrogen bonds (denaturation). These aspects will be more fully discussed in Chapters 10 and 11.

It is evident that if the molecule of deoxypentose nucleic acid consists of two interrupted polynucleotide chains held together by hydrogen bonds, the products produced by breaking all the hydrogen bonds will be relatively small in molecular weight and will probably diffuse away from each other in solution. On the other hand, the continuous structure would produce, under the same conditions, only two chains, each half the molecular weight of the original, which in view of their intertwined nature could only separate with difficulty. A considerable amount of conflicting experimental evidence has been produced in support of both views. The evidence for the discontinuous structure rests largely on the presence of secondary phosphate groups every twenty or thirty nucleotides, as evidenced by the original titration data and measurements of molecular size of molecules treated with hydrogen bond breaking reagents, *e.g.* acid, alkali or heat. The more recent titration evidence, however, does not support the existence of terminal secondary phosphate groups (see above) and the light scattering measurements of Doty and Rice[39] indicate that when the hydrogen bonds are broken by heat or low pH the molecular weight is unaltered. It would, therefore, appear that the structure more in conformity with the present experimental evidence, is the originally conceived continuous structure.

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10

THE STRUCTURE OF NUCLEIC ACIDS: 3 THE SIZE AND SHAPE AND SOME OTHER PROPERTIES OF THE DEOXYPENTOSE NUCLEATE ION IN SOLUTION

INTRODUCTION

The detailed molecular structure of the deoxypentose nucleate ion, as depicted by the Watson–Crick model, is very largely based, as has been shown in Chapter 9, on the x-ray study of concentrated gels possessing a high degree of crystallinity. Of equal importance are the studies of the nucleate ion in solution using the techniques of colloid chemistry, which have led to a knowledge of the molecular weight, size, shape and rigidity of the deoxypentose nucleate ion in solution. In contrast to the molecular weight, which will be a constant for a given preparation, the shape of the macro-ion, which is dependent on a balance of hydrogen bonding, van der Waals, inter-ionic and kinetic forces, varies with the environment. Thus changes in the shape and rigidity of the macro-ion are found to accompany changes in the ionic strength and the pH of the solution. The biological significance of these changes in the morphology of the molecule will depend on the extent to which they are transferred to the nucleoprotein. Studies of the solution properties of nucleoproteins have, so far, been less extensive than those of nucleic acids. Nevertheless, that changes of shape and rigidity of the deoxypentose nucleate ion occur with relatively small changes in the ionic environment cannot fail to be of significance in a molecule which is believed to carry the genetic code and which may replicate itself by a form of template mechanism.

Physico-chemical measurements on solutions of deoxypentose nucleic acid must be carried out under exactly specified conditions. The preparation of solutions and the determination of concentration present several difficulties. Alternative methods have from time to time been used by different workers and this may well be responsible, in part, for the conflicting results which have been reported. The sodium salt of deoxypentose nucleic acid is more readily soluble in water than in electrolyte solutions; however, it is now evident, as will be shown in Chapter 11, that an irreversible denaturation occurs when sodium deoxypentose nucleate is dissolved in water to give a nucleate solution of less than 2×10^{-4}M (6×10^{-5} g/ml.) or in solutions of sodium chloride of concentration less than 10^{-3}M. Solution in water with the subsequent addition of electrolyte is therefore not a satisfactory method if the native character of the deoxypentose nucleic acid is to be preserved; exhaustive dialysis against water is thus also to be avoided. As has been realized for some time, solution of sodium deoxypentose nucleate in water and aqueous solutions of electrolytes does not necessarily produce immediately a true molecularly dispersed solution, but one which may contain aggregates of deoxypentose nucleate ions which disaggregate only slowly. This aggregation may be aided by the presence of small amounts of impurities such as protein[1]. The presence of these aggregates may lead to two different types of error depending on the nature of the measurement to be made. If the aggregates are removed by centrifugation, as during the early stages of a sedimentation experiment or during the clarification procedure prior to light scattering measurements, the concentration of the solution with respect to the nucleate will be altered. If the aggregates are not removed automatically by some essential preliminary procedure and remain in the solution on which, for example, viscosity or other hydrodynamic measurements are made, the presence of these aggregates may cause an actual error in measurement.

It is thus to be recommended that solutions be prepared by dispersing sodium deoxypentose nucleate in a solution of sodium chloride of concentration not less than 10^{-3}M and allowing the solution to take place in the cold for several hours (for instance overnight) without excessive agitation. (The same minimum concentration applies to all uni-univalent electrolytes; for biunivalent electrolytes, *e.g.* magnesium chloride, the minimum concentration is much lower.) The rate of solution is generally more rapid in solutions of low ionic strength so that if solutions of high ionic strength are required, an initial solution should be prepared using 10^{-3}M sodium chloride and the concentration subsequently increased by adding more electrolyte. The solution should then be centrifuged at about 18,000-20,000*g* for about 1 hour. Higher gravitational fields may be used but care must be used to prevent any fractionation of the molecularly dispersed nucleate. Using a procedure of this kind Butler, Laurence, Robins and Shooter[2] have observed a marked improvement in the reproducibility of physicochemical measurements on different preparations.

The concentration of solutions prepared in this manner is not known with any accuracy. It is doubtful whether the absolute concentrations of nucleic acid solutions can be measured, because all measurements, whether dependent on phosphorus analysis or ultra-violet absorption measurements, are based ultimately on a measurement of the weight of a sample of solid sodium deoxypentose nucleate. The water content of sodium deoxypentose nucleate in equilibrium with the atmosphere is high and variable and although drying to constant weight in a vacuum at 110°C or at some lower temperature may give a reproducible standard, the water has not necessarily all been removed. A useful standard procedure adopted in many laboratories is to prepare a standard reference curve giving the relationship between ultra-violet absorption at 259 mμ and concentration. Such a standardization curve is obtained by determining the ultra-violet absorption of solutions in 4 per cent sodium chloride (a high electrolyte concentration is necessary in view of the variation of absorption with electrolyte concentration at low concentrations) prepared by weighing material dried to constant weight under a suitable standard and reproducible procedure.

Solutions of sodium deoxypentose nucleate at pH 7 in electrolyte solution may be stored at 0°C or lower for considerable periods without noticeable degradation.

THE MOLECULAR WEIGHT OF SODIUM DEOXYPENTOSE NUCLEATE

The methods which have been generally employed for the determination of molecular weights have been a combination of sedimentation coefficient with diffusion coefficient using the Svedberg equation[3], a combination of sedimentation coefficient with intrinsic viscosity using the equation of Mandelkern, Krigbaum, Scheraga and Flory[4], and by light scattering methods.

The first measurements of the sedimentation coefficient of the deoxypentose nucleate ion by Tennent and Vilbrandt[5], Cecil and Ogston[6], Stern and Atlas[7], and Kahler[8] served to demonstrate the marked concentration dependence of the sedimentation coefficient at concentrations below $2{\cdot}5 \times 10^{-3}$ g/ml. in 0·2M sodium chloride solution. These measurements were made using a cell holding a 12 mm column of solution and a Philpott–Svensson, Schlieren optical system. The lower limit of concentration of sodium deoxypentose nucleate which can be studied with such an experimental system, which is determined by the refractive index increment of the solute, is normally about 3×10^{-4} g/ml., although measurements at slightly lower concentrations have been recorded. As the value of the sedimentation coefficient is increasing rapidly with dilution in this range (see *Figure 40*) extrapolation to give the value of the sedimentation coefficient at infinite dilution cannot be made with a high degree of accuracy. Nevertheless, Kahler[8] and Ogston[9], on the basis of measurements down to a concentration of 3×10^{-4} g/ml., attempted an extrapolation assuming that the function $1/S_{20}$ (where S_{20} is the sedimentation coefficient at 20°C corrected for the viscosity of the solvent), varies linearly with the concentration. The values of S°_{20} (the value at infinite dilution) so obtained were 12·5 and 13·2S (Svedberg unit) respectively. Using a cell of similar size, Peacocke and Schachman[10] obtained measurements down to 1×10^{-4} g/ml.: in the concentration range 1 to 8×10^{-4} g/ml., $1/S^{\circ}_{20}$ was found to be a linear function of concentration and extrapolation to infinite dilution gave a value of approximately 21S for the deoxyribose nucleate from calf thymus.

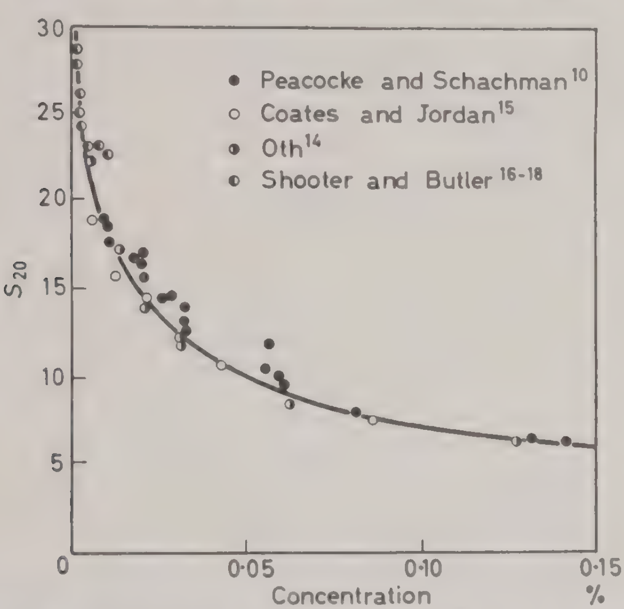

Figure 40. Variation of sedimentation coefficient of sodium deoxyribose nucleate of calf thymus with concentration

Similar values of the sedimentation coefficient at infinite dilution, higher than those originally obtained by Kahler and Ogston, have been obtained by Kahler and Shack[11], Doty, McGill and Rice[12] and by Kawade and Watanabe[13] from measurements in the concentration ranges 3 to 6×10^{-4} g/ml., 1·5 to $7 \cdot 5 \times 10^{-4}$ g/ml. and 2 to 32×10^{-4} g/ml. respectively. Kahler and Shack[11] and

Doty, McGill and Rice[12], by extrapolation of $1/S_{20}$ to zero concentration obtained the values for S°_{20} of 20S (in 0·3M potassium chloride) and 22S (in 0·2M sodium chloride) respectively. Kawade and Watanabe[13] found that the function $S^{\circ}_{20}\ (1 + [\eta]c)$, where $[\eta]$ is the intrinsic viscosity and c the concentration, varied linearly with concentration and on extrapolation of this function to zero concentration obtained the value of 23S for S°_{20}. Lower values were obtained for the deoxyribose nucleate from herring spermatozoa. The difference between the low values obtained by Kahler[8] and Ogston[9] and those obtained by Peacocke and Schachman[10], Kahler and Shack[11] and Kawade and Watanabe[13] cannot be attributed solely to the inaccuracies of the extrapolation method as the latter authors were almost certainly using better preparations of sodium deoxypentose nucleate.

It is doubtful whether measurements made on solutions having concentrations greater than about 2 to 5×10^{-4} g/ml. can be extrapolated to give a significant value at zero concentration. In order to obtain a meaningful value at infinite dilution it is necessary to make measurements on solutions that are so dilute that the molecules are for the most part independent of one another. For globular proteins, the concentration range in which meaningful measurements can be made extends to as high as 5×10^{-2} g/ml., but with nucleic acids it is much lower and 2 to 5×10^{-4} g/ml. is the upper limit. It is therefore very desirable that the methods of measurement should be so modified as to permit the study of as low concentrations as is possible.

Values of the sedimentation coefficient have been obtained at lower concentrations either by using a cell holding a 30 mm column of solution or by using an ultra-violet light absorption optical system. The former method permits measurements to be made at concentrations down to about 6×10^{-5} g/ml., while the latter method enables concentrations as low as 1×10^{-5} g/ml. to be studied with ease, and measurements have been made at concentrations as low as 1×10^{-7} g/ml. Employing a 30-mm cell, Oth[14] and later Coates and Jordan[15] determined the sedimentation coefficient of samples of deoxyribose nucleate prepared from calf thymus by the detergent method (see Chapter 2, ref. 60) in the concentration range 6·3 to $12{\cdot}6 \times 10^{-5}$ g/ml. (*Figure 40*). Extrapolation to zero concentration using the function $1/S_{20}$ gave a value of S°_{20} of 29·4S[14]. The most extensive results at low concentrations, however, have been obtained by Shooter and Butler[16–19] employing an ultra-violet light optical system. Their results (*Figure 41*) show an almost linear relationship between S_{20} and the concentration in the range 5 to 1×10^{-5} g/ml.

and extrapolation to infinite dilution gave a value of approximately 29S for S_{20}° for sodium deoxyribose nucleate prepared from calf thymus by the detergent method. This value is in good agreement with that given by Oth. For a sample of deoxyribose nucleate prepared from calf thymus by an enzymic method (see Chapter 2, ref. 63) a lower value of about 24S was obtained.

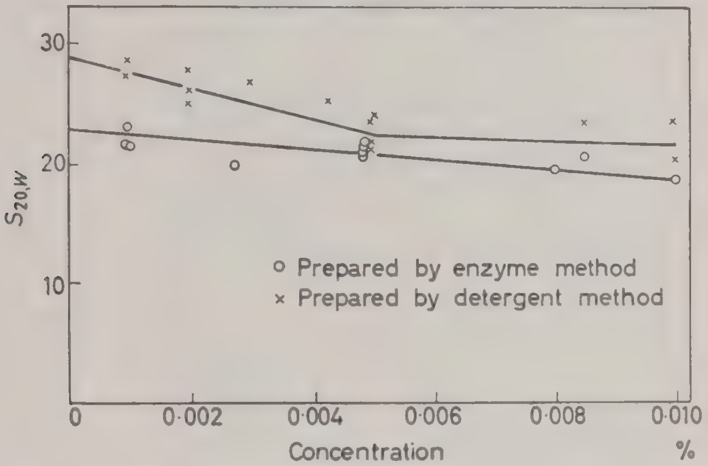

Figure 41. Variation of sedimentation coefficient of sodium deoxypentose nucleate with concentration at low concentrations in 0·2M sodium chloride

(K. V. Shooter and J. A. V. Butler: *Trans. Faraday Soc.* 1956, **52,** p. 738)

An empirical relationship between sedimentation constant and molecular weight has been determined by Doty, McGill and Rice[12] for the sodium deoxyribose nucleate of calf thymus which may well be of more general application since Barbu, Joly, Robins and Shooter[20] have shown that the same relationship holds for the deoxypentose nucleate from bacteriophage D4 of *Salmonella enteritidis*. Doty, McGill and Rice[12] determined the molecular weight, by light scattering, of deoxyribose nucleates degraded by ultrasonic degradation. From subsequent denaturation studies on the sonicates it was clear that little, if any, single-chain scission had occurred during the irradiation so that the sonicates were true homologues of the original nucleates and still possessed the double strand hydrogen bonded structure of the Watson–Crick model. Fractions in the molecular weight range $7{\cdot}4 \times 10^6$ (the original material) to 3×10^5 were obtained and the relationship:

$$S_{20}^{\circ} = 0{\cdot}063\text{M}^{0\cdot37}$$

was found to hold. This relationship may be used to estimate the molecular weight distribution of sodium deoxypentose nucleates from the sedimentation distribution obtained from sedimentation studies on dilute solutions using an ultra-violet optical system.

The influence of electrolyte concentration on the sedimentation coefficient of sodium deoxypentose nucleate has been determined by Stern and Atlas[7] for solutions of sodium chloride and by Kahler and Shack[11] for solutions of magnesium chloride. In both cases the sedimentation coefficient increases rapidly with increase of electrolyte concentration reaching a limiting value in solutions of sodium chloride at 0·2M and in magnesium chloride solutions at 0·05M (*Figure 42*).

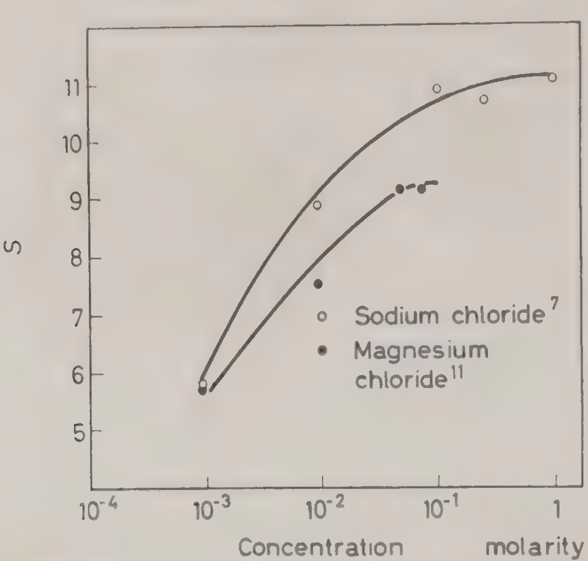

Figure 42. The influence of electrolyte concentration on the sedimentation coefficient of sodium deoxyribose nucleate

A striking feature of the sedimentation behaviour of sodium deoxypentose nucleate is the hypersharp boundary that is invariably found for a sample that is relatively undenatured, both in the presence and absence of added electrolyte at concentrations above about 1 × 10^{-4} g/ml.[10,16]. Boundaries in magnesium chloride solution are noticeably less sharp, however, than boundaries in sodium or potassium chloride solutions[11]. A typical hypersharp boundary for sodium deoxypentose nucleate is shown in *Plate 4*. It has been realized for some time that the sharpness of the sedimenting boundary is not an indication of the homogeneity of the sedimentation coefficient and hence of molecular weight. The cause of the hypersharp nature of the boundary has generally been

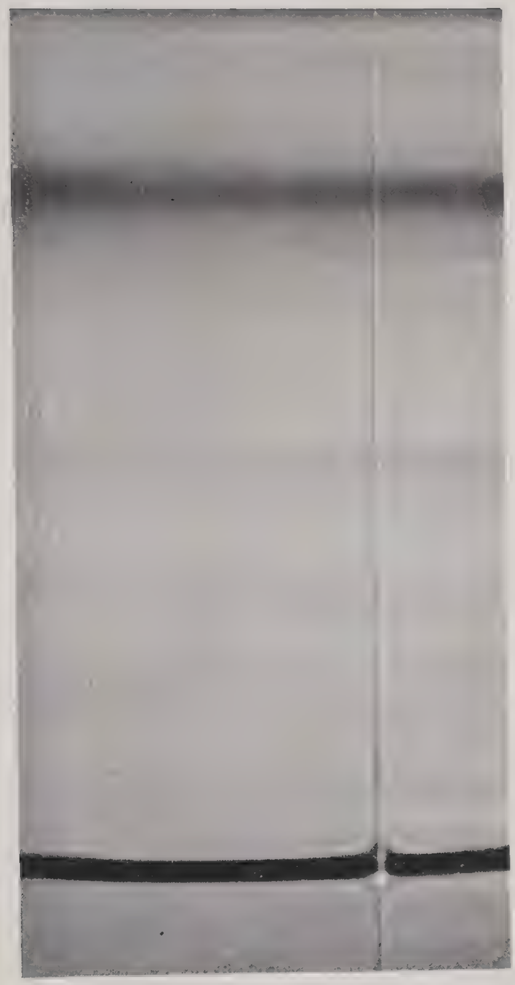

Plate 4. Typical hypersharp sedimenting boundary for sodium deoxypentose nucleate

(J. H. Coates and D. O. Jordan: *Biochim. biophys. Acta* 1959, **35** p. 309)

attributed (see, for example, Peacocke and Schachman[10]) to the marked dependence of the sedimentation coefficient on the concentration, so that the boundary is under the influence of a self-sharpening effect. However, this view requires modification, since it has been shown by Coates and Jordan[15] that the synthetic boundary formed between 5 and 10 × 10^{-4} g/ml. solutions in 0·2M sodium chloride for which the sedimentation coefficients are similar (*Figure 40*) is just as hypersharp as that between zero concentration and 5 × 10^{-4} g/ml. in the same solvent. If the concentration dependence hypothesis is correct, it would be expected that the former boundary would show normal spreading because of the small variation of sedimentation coefficient across the boundary, whereas the latter would be hypersharp. Although no satisfactory explanation of this behaviour has been put forward, Coates and Jordan[15] consider the formation of hypersharp boundaries to be related to the dependence of the charge, carried by the nucleate ion, on the concentration. At concentrations below 10^{-4} g/ml. the boundaries show a marked degree of spreading and Shooter and Butler[16–18] have shown that this is to be attributed to a true physical heterogeneity of the nucleic acid sample and not to an instability of the boundary. From the shape of the boundary, the sedimentation coefficient distribution at infinite dilution may be calculated[17–19], and for a sample prepared by the detergent method, the sedimentation coefficient is found to range from 12S to about 40S (see Chapter 3, *Figures 1* to *3*, pp. 39 to 41). The value of 29S referred to above was calculated from the rate of movement of the centre of the boundary and therefore represents a weight average value.

The concentration dependence of the sedimentation coefficient appears to vary with the nature and hence the source of the nucleic acid. The sodium deoxypentose nucleate from bacteriophage D4 of *Salmonella enteritidis*, for example, exhibits such a marked variation of sedimentation coefficient with concentration that extrapolation to infinite dilution is not possible even when measurements have been made down to 1 × 10^{-5} g/ml. Measurements at even lower concentrations, 10^{-5} to 10^{-7} g/ml., were made by Goodgal, Rupert and Herriott[21] on the deoxypentose nucleic acid of the transforming principle of *Haemophilus influenzae* using the partition cell method. The sedimentation coefficient in this range of concentration was found to be 27 ± 2·5S.

The determination of the diffusion coefficient of sodium deoxypentose nucleate has proved difficult for a variety of reasons. Because of the marked concentration dependence of the diffusion coefficient an unsymmetrical (skewed) boundary is obtained[8] and there may also be an apparent movement of the boundary[22]. The only extensive investigations which have been made are those of Cecil and Ogston[6] and of Kahler[8]. The former authors obtained the value of $8{\cdot}1 \times 10^{-8}$ cm^2/sec at infinite dilution in 0·2M sodium chloride at 20°C, while the latter author gave the value of $4{\cdot}5 \times 10^{-8}$ cm^2/sec at infinite dilution in a phosphate buffer (pH 7·4, ionic strength 0·3) at 20°C. Comparison of these results is difficult in view of the different nature of the solvent and is made more difficult by the fact that while Cecil and Ogston, using the Gouy method, observed a decrease of the diffusion coefficient with increase in concentration, Kahler, using the Schlieren method, observed an increase. The latter form of concentration dependence has been confirmed by Howard[23]. Coates[24] has attempted to measure the diffusion coefficient using a Rayleigh optical system, but has found that the formation of an initially sharp boundary by the usual flushing technique is very difficult in view of the relatively high viscosity of even dilute solutions of sodium deoxypentose nucleate. Nevertheless, the approximate value of 3×10^{-8} cm^2/sec was recorded for a concentration of 8×10^{-4} g/ml. in phosphate buffer at pH 7·0 and ionic strength 0·2 at 25°C. This value is in reasonable agreement with that of 2×10^{-8} cm^2/sec given by James[25] for solutions in water and 0·2M sodium chloride at infinite dilution using a method designed for solutions of concentration 1×10^{-5} g/ml. and lower. A value of 3×10^{-8} cm^2/sec has also been obtained for the deoxypentose nucleic acid of the transforming principle of *Haemophilus influenzae*[21]. It would thus appear from these preliminary investigations that the diffusion coefficient of satisfactory preparations is of the order of 2 to 3×10^{-8} cm^2/sec. Combining the value of 2×10^{-8} with that of 24S for the sedimentation coefficient using the Svedberg equation, both values being obtained with a deoxyribose nucleate from calf thymus prepared by an enzymic method, gives a value of the molecular weight of 6×10^6. Combination of the values 3×10^{-8} for the diffusion coefficient and 29S for the sedimentation coefficient for a preparation by the detergent method gives a value of 5×10^6.

The difficulties experienced in obtaining reliable values of the diffusion coefficient have led to the combination of the sedimentation coefficient with other data to give values of the molecular weight. This procedure generally involves an assumption concerning the shape and flexibility of the deoxypentose nucleate ion. The method most frequently used is the combination of sedimentation coefficient with intrinsic viscosity data using the equation of Mandelkern, Krigbaum, Scheraga and Flory[4]. The intrinsic viscosity yields information concerning the size of the molecule in solution, and in c.g.s. units represents approximately the effective volume of the molecule. The values that have been obtained for sodium deoxyribose nucleate from calf thymus at zero shear by different workers vary over a wide range. While Pouyet[26], Conway and Butler[27], Jordan and Porter[28], Reichmann, Rice, Thomas and Doty[29], and Eisenberg[30] all record values in the range 5·0 to 5·5 × 10^3 c.g.s. units, the values given by Butler, Laurence, Robins and Shooter[2] for six different specimens of this nucleate show an almost two-fold variation, the values ranging from 2·9 to 5·0 × 10^3 (see *Table 14*, p. 192). Cavalieri, Rosoff and Rosenberg[31] record values in the range 3·4 to 5·7 × 10^3 for various preparations and Doty, McGill and Rice[12] give a value of 6·9 × 10^3 for a sample having a light scattering molecular weight of 7·4 × 10^6. The latter authors have also shown from studies on sonic fragments, that the relationship

$$[\eta] = 1{\cdot}45 \times 10^{-4} M^{1{\cdot}12}$$

holds for true homologues in the molecular weight range 3 × 10^5 to 7·4 × 10^6.

In order to apply the equation of Mandelkern, Krigbaum, Scheraga and Flory[4], it is necessary to make an assumption concerning the shape of the nucleate ion in solution. The equation may be written in the form :

$$M^{2/3} = \frac{S^\circ[\eta]^{1/3}}{\beta} \cdot \frac{\eta_0 N}{(1 - \bar{V}\rho)}$$

where β varies from 2·12 × 10^6 for an axial ratio of 1, to 2·6 × 10^6 for a statistical coil and to 3·6 × 10^6 for a rigid rod of very high axial ratio. That this relationship holds for deoxypentose nucleic acids has been demonstrated by Barbu, Joly, Robins and Shooter[20] as evidenced by a straight line relationship between S° and $[\eta]^{1/3}$ for several different samples of native deoxypentose nucleate. Doty, McGill and Rice[12], however, interpret their results on the viscosity, sedimentation and light scattering of various homologous sonic fragments in terms of a variation of β from 2·56 at a molecular weight of 3 × 10^5 to 3·29 at 8 × 10^6, thus indicating a change in the axial ratio with molecular weight. The majority of molecular weight calculations using this equation have nevertheless been made using the value of 2·6 × 10^6 as the value for β which makes the assumption that the nucleate ion is behaving as a statistical coil. It is very doubtful whether the nucleate ion can ever become sufficiently flexible to adopt this configuration and the application of the equation to nucleic acids cannot be regarded as an entirely rigorous procedure. Calculations using this equation have yielded values[2, 10, 32, 33] for various preparations, in the range 6 to 12 × 10^6 (see *Table 11*). The most extensive results are those of Butler, Laurence, Robins and Shooter[2], who give values in the narrower range of 8·5 to 10 × 10^6. Mathieson and Porter[32] have also calculated the molecular weight from the viscosity and the orientation angle (obtained from streaming birefringence measurements) from data obtained with concentrated solutions using the equation of Peterlin[34]. The molecular weight, so calculated, was found to be either 1·5 or 4·5 × 10^6 depending upon whether the macro-ion was assumed to be rigid or flexible respectively. The values obtained by these methods of determining the molecular weight are summarized in *Table 11.*

Table 11. The molecular weight of various samples of sodium deoxypentose nucleate determined from sedimentation (S) and diffusion (D) coefficients and intrinsic viscosity ($[\eta]$)

Source	*Method of preparation*	*Method*	$M \times 10^{-6}$	*Reference*
Calf thymus	Enzyme	S° and D°	6	16–19, 25
Calf thymus	Detergent[2]	S° and D*	5	14, 17, 24
Haemophilus influenzae	Not given	S° and D°	15	21
Calf thymus	Signer[3]	S° and $[\eta]$	8·5	10
Calf thymus	Enzyme[1]	S° and $[\eta]$	6·6 ; 10·5	33
Calf thymus	Detergent[2]	S° and $[\eta]$	9·4 ; 11·6	33
Calf thymus	Chloroform[4]	S° and $[\eta]$	2·2	32
Calf thymus	Not given	S° and $[\eta]$	{8·5 ; 9·0 ; 9·5 ; 10·0 ; 10·8}	33
Phage D4 of *Salmonella enteritidis*	Wahl[35]	S° and $[\eta]$	26	20

* Not extrapolated to infinite dilution

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4. Gulland, J. M., Jordan, D. O. and Threlfall, C. J. *J. chem. Soc.* 1947, p. 1129

The most extensive data on the molecular weight of various preparations of sodium deoxypentose nucleate, however, have been obtained from measurements of the light scattering of solutions. The scattering of light by charged polymers is dependent on the electrostatic repulsion between the macro-ions, the intensity of the scattered light and angular distribution both being affected. However, by extrapolating to infinite dilution and by making measurements on solutions containing added electrolyte, the difficulties of interpretation caused by the presence of a charge on the polymer may be largely overcome. The accuracy of light scattering measurements on solutions of sodium deoxypentose nucleate has been estimated as ± 12 per cent by Doty and Bunce[36] and as ± 15 per cent by Sadron, Pouyet and Vendrely[37]. The early work of Oster[38] and of Smith and Sheffer[39] has been greatly extended by Doty and Sadron and their collaborators and the

Table 12. The molecular weight of various samples of the sodium deoxyribose nucleate of calf thymus determined from light scattering measurements

Method of preparation and sample number	$M \times 10^{-6}$	*Reference*
Chloroform	4·4	39
Chloroform (Gulland preparation)	(4·0), 3·5	(36), 29
Signer (SVII)	(6·7), 5·9	(36), 29
Signer (Doty preparation)	(4·0), 3·5	(36), 29
Signer (Doty preparation)	7·7	40, 41
Detergent (SBI)	5·85	42, 29
Detergent (SCI)	6·6	42
Signer (V1)	6·85	42, 29
Detergent (SBII)	8·2	42
Detergent (Dounce preparation)	4·7	29
Chargaff[1]	4·65	29
Signer (Brown preparation)	6·0	43
Chloroform (Rowen preparation)	4·5	44, 45
Signer (Katz preparation)	8·0	46
Signer (CV49)	4·6	37, 47–49
Signer (SVIII)	6·0	37, 47–49
C.R.M.* (CV3)	1·9	49
Signer (CV42)	4·0	37, 47–49
Signer (SV)	6·0	37, 47–49
Signer (SXII)	11·6	49, 50
Detergent (CV9)	15·0	37, 47–49
Signer (CV5)	16·5	37, 47–49
Signer (SX)	3·8	49, 50
Signer (I69; CV69; CV71)	6·0	37, 48
Signer (CV62; CV64)	13·0	37, 48
Signer (I62)	14·0	37, 48
Not given	3·0, 3·3, 4·0, 17	2
Detergent	7·4	12
Chloform	4·4, 7·3	58

* Prepared by Centre de Recherches sur les Macromolecules, Strasbourg, France, exact method not specified

1. Chargaff, E. and Zamenhof, S. *J. biol. Chem.* 1948, **173**, p. 327

results of measurements on various preparations of sodium deoxypentose nucleate from calf thymus and from other tissues are summarized in *Tables 12* and *13*. The three values in parenthesis were calculated by Doty and Bunce[36] using the value of 0·160 for the specific refractive index increment obtained by Tennent and Vilbrandt[5]; a value of 0·188 for a better nucleic acid preparation was subsequently obtained by Reichmann, Rice, Thomas and Doty[29], who recalculated the Doty and Bunce results on this basis. It would appear that this figure of 0·188 has been used in most subsequent measurements although Cavalieri, Rosoff and Rosenberg[31] used the value 0·191 and a somewhat higher value of 0·201 has been reported[54].

Table 13 The molecular weight of various samples of sodium deoxypentose nucleate from different sources determined from light scattering measurements

Source	*Method of preparation*	$M \times 10^{-6}$	*Reference*
Escherichia coli	Detergent	7·0	51
Arbacia lixula	Chargaff[1]	2·3	43
Escherichia coli	Chargaff[1]	9·1	43
Avian tubercle bacillus	Chargaff[1]	14·4	43
Herring spermatozoa	Signer	6·0	52
Trout spermatozoa	Mirsky	6·0	37, 48
Chicken erythrocyte	Signer (CV51)	11·6	37, 48, 49
Chicken erythrocyte	Signer (CV78)	15·0	37, 48
Chicken erythrocyte	Not given	4·2	53
Pneumococcus	Not given	7·7	53

[1] Chargaff, E., Lipshitz, R. and Green, C. *J. biol. Chem.* 1952, **195**, p. 155; Gandelman, B., Zamenhof, S. and Chargaff, E. *Biochim. biophys. Acta* 1952, **9**, p. 399; Chargaff, E., Zamenhof, S., Brawerman, G. and Kerin, L. *J. Amer. chem. Soc.* 1950, **72**, p. 3825; Zamenhof, S., Brawerman, G. and Chargaff, E. *Biochim. biophys. Acta* 1952, **9**, p. 402; Snellman, O. and Widström, G. *Arkiv. Kemi Min. Geol.* 1945, **19A**, No. 31

Excluding the values greater than 11×10^6 and those obtained using the chloroform-gel method for separating the nucleic acid and protein, the majority of the remaining values for the nucleate prepared from calf thymus (*Table 12*) fall approximately within the range[37, 53] 5·8 to $7{\cdot}7 \times 10^6$. A narrower range of molecular weights from this method is not to be expected in view of the estimates of the accuracy of the method which have been given above. The high values of the molecular weight given in *Table 12* are approximately double the lower values[37], and the possibility of aggregation to dimers has been tentatively suggested[37]. However, Butler, Laurence, Robins and Shooter[2] have shown that the effectiveness of the preliminary clarification of solutions prior to the measurement of light scattering is very sensitive to the concentration of the solutions. Thus centrifugation at 20,000g for 18 hours of a 0·1 per cent solution yields a value for the molecular weight of more than 20×10^6, while clarification of a 0·04 per cent solution of the same nucleic acid sample yields a value of 3×10^6. This improved clarification is to be attributed to the lower viscosity of the more dilute solutions. The material responsible for the high turbidity has been shown not to be deoxypentose nucleic acid and is therefore a true impurity and furthermore its presence affects neither the sedimentation coefficient nor the intrinsic viscosity of the sample. It would appear therefore that any consideration of the high molecular weight values should await a more careful examination of the purity of the samples and the preparation of the solutions.

There have been few comparisons of the values of the molecular weight determined by different methods on the same sample. Such comparisons must be carried out not only with due regard to the type of molecular weight average that a particular method yields, but also to the sensitivity of the method to changes in molecular shape and to other assumptions implicit in the formula used. Combination of sedimentation and diffusion coefficients gives an average molecular weight close to, but not equivalent to, a z-average molecular weight, while combination of sedimentation coefficient and intrinsic viscosity will give an average approximating to a weight average. Light scattering, in general, gives a weight average molecular weight, but is to some extent dependent on the molecular weight distribution[55, 56] (see below).

A direct comparison of the molecular weights determined by light scattering and from sedimentation coefficient and intrinsic viscosity has been made by Butler, Laurence, Robins and Shooter[2] and can also be obtained from the data of Doty, McGill and Rice[12] for homologous sonic fragments. The results are given in *Table 14*.

The discrepancies between the two methods of calculating molecular weights have been attributed to the failure of the light scattering theory when applied to very long rods and to the effect of heterogeneity on both types of measurement[2]. Doty[56], however, has shown that the heterogeneity factors in the Mandelkern, Krigbaum, Scheraga and Flory relationship remain almost constant over the range where M_w/M_n varies from 1·5 to 5. It is usual in light scattering studies of solutions of sodium deoxypentose nucleate to assume that $M_z : M_w : M_n = 3 : 2 : 1$ since this avoids the necessity of making measurements at very low scattering angle which is experimentally very difficult, if not impossible. Doty[56] has demonstrated that a very broad molecular weight distribution in which M_w/M_n is greater than 5 would be necessary to raise the light scattering molecular weight values to those calculated from viscosity and sedimentation. Values of the ratio M_w/M_n have not yet been determined experimentally although Schachman[57] has estimated it to be 3 : 1. Indirect evidence is available from a study of the effect of acid on the shape of the deoxypentose nucleate ion[29].

Table 14. The molecular weights of various samples of the sodium deoxyribose nucleate from calf thymus determined from light scattering (M_L) and from sedimentation coefficient and intrinsic viscosity ($M_{S,\eta}$)

Specimen number	S°_{20} Svedberg units	$[\eta] \times 10^{-3}$ c.g.s. units	$M_{S,\eta} \times 10^{-6}$	$M_L \times 10^{-6}$	*Reference*
31C	27	3·5	10·0	3·0	2
33	25	2·9	8·5	3·3	2
36	28	3·34	10·8	3·3	2
40	22	5·0	9·0	4·0	2
34	25	3·4	9·0	3·3	2
38	23	4·7	9·5	17	2
0*	22·0	6·9	10·4	7·4	12
1*	12·2	3·73	3·2	3·4	12
5*	9·0	0·535	7·6	7·0	12
20*	7·75	0·312	4·6	4·5	12
60*	6·9	0·177	2·9	3·0	12

* Time of sonic irradiation in minutes

In acid solution the size of the nucleate ion decreases considerably compared with that in neutral solution whereas there is no change of molecular weight. This result is interpreted by Doty as indicating that the ratio M_w/M_n cannot be far from 2 and certainly cannot be as great as 5.

The discrepancies between the two methods of calculating the molecular weight most probably reside in the difference between the hydrodynamic model assumed in the Mandelkern, Krigbaum, Scheraga and Flory equation and that of the deoxypentose nucleate ion. It is clearly evident that further studies on the heterogeneity of samples at infinite dilution are needed to clarify the position or alternatively a study of sharper fractions obtained by chemical or physical fractionation.

Few other methods of determining the molecular weight of nucleic acids have been employed, indeed few others exist. However, Fluke, Drew and Pollard[59] have obtained a value of approximately 6×10^6 for the deoxypentose nucleic acid from the pneumococcus transforming principle by analysis of the activity remaining after bombardment of a specimen with 2 MeV electrons and 3·8 MeV deuterons. Mathieson and Porter[32] have also calculated the molecular weight of calf thymus deoxyribose nucleate from the intrinsic viscosity and rotary diffusion constant to be 3×10^6.

In conclusion it may be stated that there is fairly good evidence that the weight average molecular weight as determined by light scattering for good preparations of sodium deoxyribose nucleate from calf thymus lies between 6 and 8×10^6. Preparations giving lower values have probably been degraded during preparation. Insufficient determinations have been made on deoxypentose nucleates from other tissues to be able to draw a similar conclusion concerning their molecular weights. Certainly the evidence is insufficient to support the contention of Sadron, Pouyet and Vendrely[37] that the deoxypentose nucleic acids of all tissues possess the same molecular weight. Such an hypothesis requires evidence not only of identical molecular weights, but also of molecular weight distribution. Furthermore it is to be recalled that the physical heterogeneity of the nucleic acids may have real genetic significance (see Chapter 3) and it is probable that only the molecular weights of analogous fractions, prepared by either physical or chemical fractionation, may be capable of comparison.

THE MOLECULAR CONFIGURATION OF SODIUM DEOXYPENTOSE NUCLEATE IN SOLUTION

The dimensions of the Watson and Crick structure as at present accepted (see Chapter 9, p. 167) show an internucleotide spacing of either 2·6 Å or 3·3 Å, depending upon whether the crystalline sodium deoxypentose nucleate is in the A or B form, and a diameter of 18 Å. Assuming that the Watson and Crick structure is maintained in solution, and considering a molecular weight of 7×10^6, the length of the macro-ion will be approximately 30,000 Å. Although the hydrogen bonding between the nucleotides in the double helix will greatly increase the rigidity of the macro-ion compared with that of a synthetic polyelectrolyte, it is nevertheless unlikely that the macro-ion will remain completely straight and rigid in solution in view of the very high axial ratio that would result[10, 60].

Information on the shape and size of the deoxypentose nucleate ion in neutral solution has been obtained from a variety of different experimental techniques including streaming birefringence, viscosity, light scattering, and, to a lesser extent, sedimentation. The interpretation of the results of these measurements may be considered in two steps. Firstly, the determination of the general shape of the macro-ion and secondly, the determination of the dimensions assuming a particular hydrodynamic model. The models most generally assumed have been the rigid rod and the statistical coil. The interpretation is made difficult by the unknown heterogeneity of the preparations and the consequent uncertainty in the analysis of the experimental data.

The axial ratio of the deoxypentose nucleate ion in solution was first determined by Signer, Caspersson and Hammarsten[61] from viscosity and streaming birefringence measurements and a value of 300 obtained. Tennent and Vilbrandt[5], Cecil and Ogston[6], and Kahler[8] have determined the frictional ratio from sedimentation and diffusion measurements and thence, using the equation of Perrin[62], calculated the value of the axial ratio. The values obtained were 120, 170 to 400 and 284 respectively for different preparations. The axial ratio may also be calculated from a knowledge of the sedimentation coefficient, intrinsic viscosity and molecular weight using the equation of Mandelkern, Krigbaum, Scheraga and Flory[4] (see p. 187). It has already been shown (p. 187) that the function β, in this equation is dependent upon the axial ratio and Peacocke and Schachman[10], using the table of values compiled by Scheraga and Mandelkern[4], have obtained the tentative value of 300 for the axial ratio of the equivalent prolate ellipsoid which will be identical with that of the real particle if the latter were rigid and impermeable to solvent. Mathieson and Matty[63], employing the equation of Simha[64] for rigid rods, have calculated the axial ratio from the intrinsic viscosity and obtained the value of 416. From the rotary diffusion coefficient obtained from streaming birefringence measurements, and using the equation of Perrin[65], Goldstein and Reichmann[66] have calculated the axial ratio for three high molecular weight preparations; the values obtained ranged from 354 to 755. All the values of the axial ratio recorded above, which have been obtained for neutral solutions of sodium deoxypentose nucleate containing sodium chloride (generally 0·2M), are subject to not inconsiderable errors as the axial ratio is generally very sensitive to variations in the experimental quantities measured. Although the values obtained by the several methods are all of the order of a few hundreds, they are all subject to the criticisms that either a rigid rod model has been assumed or that the molecular volume of the dry molecule has been used in the calculation. Both these assumptions have been criticized by Reichmann, Rice, Thomas and Doty[29], on the grounds that the rigid rod model would not give rise to the scattering envelope observed for sodium deoxypentose nucleate and that the nucleate ion is known to be considerably hydrated in solution. These authors consider that a preferable model is a solvent immobilized ellipsoid which contains the nucleate ion and solvent; the volume of the latter being about 10^3 times the volume of the nucleate ion. The axial ratio of such a model which would produce the observed values of the rotary diffusion constant is about 10, a value very much lower than those recorded above.

The most extensive data on the configuration of the nucleate ion in solution have been obtained from light scattering measurements. The interpretation of the data for the angular distribution of the scattered light, however, is not easy and involves assumptions concerning both the heterogeneity of the solution and the nature of the appropriate hydrodynamic model. Oster[67] interpreted his results in terms of a rigid rod, while Smith and Sheffer[39] considered that a statistical coil model conformed more with their data. Doty and Bunce[36] considered a variety of possible models including that of a lightly branched or cross-linked polynucleotide. The more extensive light scattering data of Reichmann, Varin and Doty[41], Reichmann, Rice, Thomas and Doty[29] and of Rowen[45] have, in general, indicated that the deoxypentose nucleate ion in solution approximates to neither a rigid rod nor a statistical coil, but that its behaviour lies between these two extremes. This intermediate configuration has been described by Rowen[45] as a slightly flexible rod and by Reichmann, Rice, Thomas and Doty[29] as a stiff coil. It would appear from the following discussion that the latter description more faithfully describes the behaviour of the nucleate ion in solution.

The molecular dimensions for three high polymer weight preparations are given in *Table 15*[29, 66]. The radius of gyration, which is the sum of the products of the mass of each segment in the molecule and the square of its distance from the centre of gravity, have been calculated directly from the Zimm plots of the light scattering data. These quantities are listed as $\bar{R}_g$ in *Table 15* and are of the order of 2000 Å for all three samples, thus indicating that the ions are extremely large for their weight and are therefore highly extended. The shape of the complete reciprocal scattering envelope (see *Figure 43*) depends on both the shape of the scattering particle and, although to a lesser extent, on the molecular weight distribution (see below). Reichmann, Rice, Thomas and Doty[29] consider that, of the simple hydrodynamic models, that which best conforms with the observed envelope is a random coil, a conclusion also reached by Eisenberg[30] from viscosity measurements on dilute solutions. The values of the root-mean-square end-to-end distance, $\sqrt{\bar{R}^2}$, given in *Table 15* have been calculated from the light scattering data on this basis. This quantity is a z-average dimension, in that it is an average dimension of the molecules corresponding to a z-average molecular weight, which would be higher than the weight average molecular weights given in *Table 15*.

Table 15. Molecular constants and dimensions for preparations of the sodium deoxyribose nucleate from calf thymus

Preparation method and sample	M $\times 10^{-6}$	$\sqrt{\bar{R}^2}$ Å	$\sqrt{\bar{R}_g^2}$ Å	L Å	θ sec^{-1}	$2a$ Å	$[\eta]\times 10^{-3}$ c.g.s. units	$R_{[\eta]}$ Å
Signer (SVIII)	5·90	5400	2200	28000	21·2	10900	5·1	5240
Signer (V1)	6·85	5030	2050	33000	4·64	19000	4·8	5400
Detergent (SB1)	5·85	4950	2020	28000	18·2	11200	5·34	5300

M = molecular weight from light scattering.
$\sqrt{\bar{R}^2}$ = mean end-to-end distance from light scattering.
$\sqrt{\bar{R}_g^2}$ = radius of gyration.
L = contour length assuming 2 nucleotides every 3·3 Å
θ = rotary diffusion coefficient.
$2a$ = length of major axis from θ.
$[\eta]$ = intrinsic viscosity at zero shear.
$R_{[\eta]}$ = mean end-to-end distance from viscosity.

(From M. E. Reichmann, S. A. Rice, C. A. Thomas and P. Doty: *J. Amer. chem. Soc.* 1954, **76**, p. 3048)

The values of the mean end-to-end distance, $R_{[\eta]}$, given in the last column of *Table 15* have been calculated[29] from the values of the intrinsic viscosity at zero shear using the equation of Flory and Fox[68]. This equation is based on a random coil hydrodynamic model and also assumes that the molecular weight distribution is not wide enough to introduce significant errors. The values obtained are in reasonable agreement with those of $\sqrt{\bar{R}^2}$ obtained from the light scattering data.

In *Table 15* are also given the values of the rotary diffusion coefficient and of the length of the major axis calculated therefrom. These values have been obtained from measurements of the streaming birefringence of sodium deoxyribose nucleate solutions by Goldstein and Reichmann[66] assuming that the molecular volume is that of the dry molecule. Similar calculations had been made previously by Schwander and Cerf[69] who obtained a value of 36·6 sec^{-1} for the rotary diffusion coefficient from which Schwander[70], assuming a value of 10 Å for the molecular diameter calculated the length of the major axis to be 8000 Å. Mathieson and Matty[63] have also calculated the length from the rotary diffusion coefficient and the intrinsic viscosity, assuming a rigid rod model, and obtained a value of 9360 Å. Although the value of a, the length of the semi-major axis, obtained by Goldstein and Reichmann (*Table 15*) are very close to the values of $\sqrt{\bar{R}^2}$, as might be expected, since both quantities are similarly related to the radius of gyration, the agreement is regarded as fortuitous[29] in view of the different hydrodynamic models assumed in the two calculations. The length of the semi-major axis was calculated assuming a rigid rod (*i.e.* an ellipsoid of high axial ratio) whereas $\sqrt{\bar{R}^2}$ was calculated on the basis of a random coil.

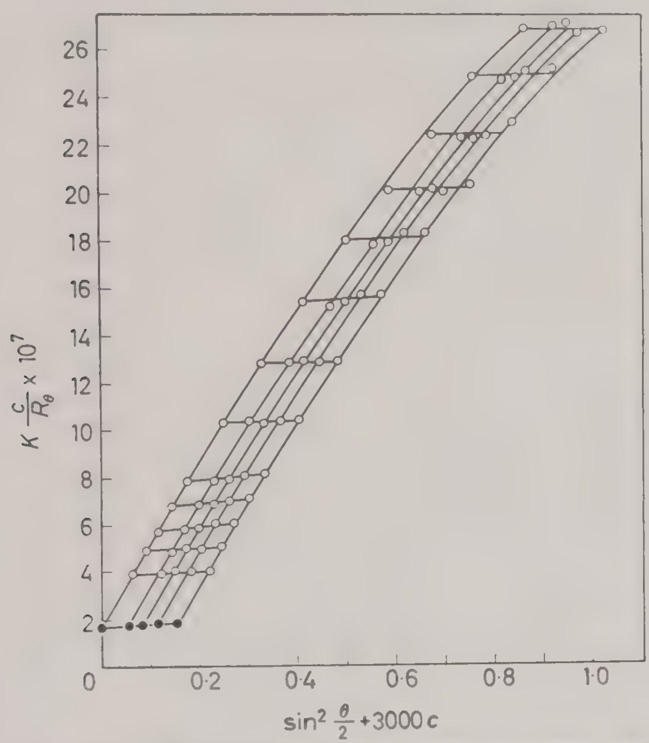

Figure 43. Light scattering data for sodium deoxyribose nucleic acid in 0·2M sodium chloride

(M. E. Reichman, S. A. Rice, C. A. Thomas and P. Doty: *J. Amer. chem. Soc.* 1954, **76**, p. 3050)

As has been shown, the shape of the complete reciprocal scattering envelope corresponds most closely, but not exactly, to the scattering envelope of a random coil. However, the downward curvature of the reciprocal scattering envelope (*Figure 43*) could arise either from an excessive heterogeneity in the molecular size and weight of the random coils or from a stiffness in random coils which possess a narrow molecular weight distribution. If an attempt is made to explain the deviation as due to a wide molecular weight distribution, it is found[29] that the unusually high values of $M_z/M_w = 2$ and $M_w/M_n = 6$ are necessary to characterize the heterogeneity. If it is assumed that a narrower molecular weight distribution such that $M_w/M_n = 2$ occurs, then it can be calculated that about half the particles are too stiff to fold into the shape of random coils.

Attempts have been made theoretically to deal with the problem of the stiff coil and Peterlin[71–73] has developed a theory in which the shape of the molecule is related to the stiffness of the coil by a parameter termed the ‘ persistence length ’ or ‘ Porod unit ’ (defined as the average value of the projection of an infinitely long thread on the tangent at one end of the thread). By adopting the ‘ worm-like ’ chain model of Kratky and Porod[74], Peterlin succeeded in relating the degree of downward curvature in the reciprocal envelope to the number and length of Porod units with which the macro-ion may be represented. Moreover, when the number of Porod units has been determined for a sample of known molecular weight (M_z) the value of the mass per unit length, M/L, can be determined. Peterlin[73] and Doty[56] have analysed the light scattering data on the basis of this theory. Doty, using the results of Rice and Doty[42], has shown that the scattering envelopes can be closely approximated by the curve calculated from Peterlin's theory using a value of 100 for the number of Porod units in the nucleate ion. The Porod unit is then found to be 500 Å and this yields a value of 230 molecular weight units per ångstrom for M/L. This compares favourably with the value of 200 required by the Watson–Crick model and offers support for the contention that the observed curvature in the reciprocal envelopes does arise from chains having a narrow molecular weight distribution and of high extension and not from an extremely broad molecular weight distribution. The length of 500 Å for the Porod unit indicates an exceptionally low degree of coiling which is in conformity with the stiff random coil model.

Of considerable relevance to the problem of the correct hydrodynamic model for the deoxypentose nucleate ion in solution, is the flexibility of the ion. It is well known that changes in the ionic environment can bring about very marked changes in the shape of randomly coiled synthetic polyelectrolytes in solution. If the deoxypentose nucleate ion were to behave as a flexible chain, it would be fully expected that similar changes in shape, as revealed by measurements of viscosity, streaming birefringence and light scattering, would take place on changing the ionic environment in solution. However, it would also be expected that, if the hydrogen bonded double helix structure persisted in solution, this would produce a considerable stiffening of the nucleate macro-ion in solution as compared with a synthetic poly-ion or a single polynucleotide chain.

Measurements of viscosity and streaming birefringence on solutions of sodium deoxypentose nucleate containing no added electrolyte have not been found to be reproducible (see, for example, Sadron[75]). This lack of reproducibility will be due to a denaturation of the nucleate ion (see Chapter 11) which is accelerated by an increase of temperature and retarded or prevented by the presence of electrolyte. However, by extrapolating the viscosity–time curves to zero time, Kurucsev[76] has recently been able to obtain reproducible values of the initial viscosity of very dilute solutions of sodium deoxypentose nucleate in water. These viscosity values will refer to the denatured and not the native form of the nucleate. In view of the difficulty of making measurements on solutions free of electrolyte, it has been usual to compare the properties of solutions in very dilute sodium chloride solutions (about 4×10^{-4}M) with those in more concentrated electrolyte. The viscosity of neutral sodium deoxypentose nucleate solutions in water or dilute electrolyte is very high and characterized by a high dependence on rate of shear (non-Newtonian viscosity). The addition of electrolyte to solutions produces a marked fall in the viscosity[77–80], which was first explained[79] as a reversible depolymerization of the deoxypentose nucleate. However, acid–base titration of the nucleate in water and in 1M electrolyte solution[81] showed that there was no liberation of acid and base groups and that the hydrogen bonded structure remained intact; furthermore light scattering studies have shown that the increase in ionic strength does not affect the molecular weight but only the shape of the nucleate ion[29]. The depolymerization explanation is thus untenable. Jordan[82], following the analogy of solutions of some polyelectrolytes, attributed the fall in viscosity to a reduction in the asymmetry of the macro-ions on the addition of sodium chloride. However, the intrinsic viscosity at zero shear shows only a small variation with ionic strength (see below), a result which led Conway and Butler[27, 83], and Butler, Conway and James[84] to the conclusion that the high viscosity of sodium deoxypentose nucleate in water is predominantly due to electrostatic interaction between the molecules. The sharp decrease in the viscosity on the addition of electrolyte is then due to the reduction of this interaction.

The effect of ionic strength on the intrinsic viscosity calculated from viscosity measurements at zero shear has been determined by several authors with somewhat conflicting results. Pouyet[26], Conway and Butler[27], Butler, Conway and James[84] and Eisenberg[30] conclude from their measurements that the η_{sp}/c against concentration of nucleate curves for different electrolyte concentrations extrapolate to the same value of the intrinsic viscosity at zero concentration. From this result these authors have concluded that

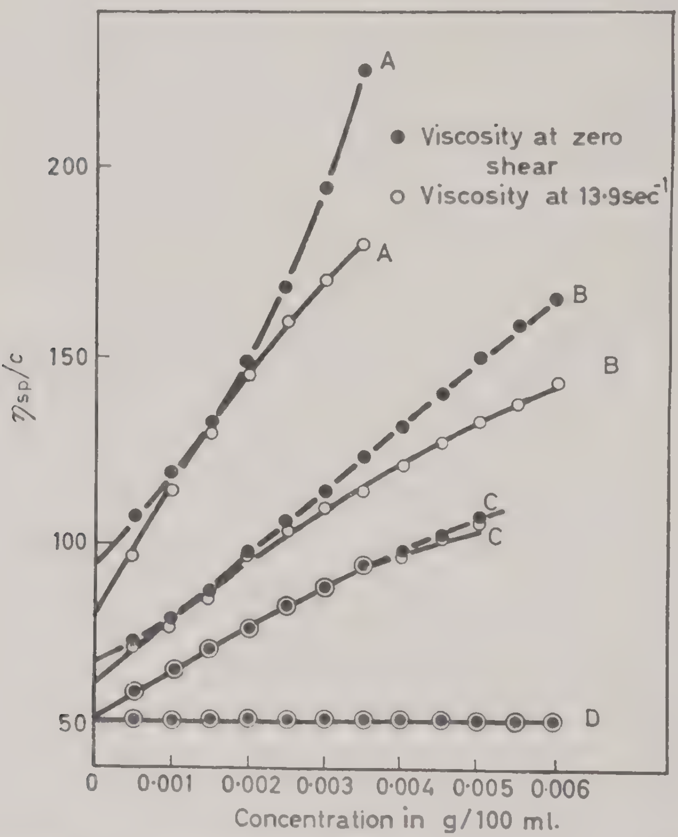

Figure 44. Variation of intrinsic viscosity with concentration of sodium deoxypentose nucleate at different concentrations of sodium chloride[85]

A, 4×10^{-4}M sodium chloride; B, 1×10^{-3}M sodium chloride; C, 4×10^{-3}M sodium chloride; D, 1×10^{-1}M sodium chloride the intrinsic viscosity is independent of the electrolyte concentration and hence that the nucleate ion is inflexible and rigid. Careful studies by Jordan and Porter[28, 85], and by Cavalieri, Rosoff and Rosenberg[31] at slightly lower concentrations, however, show a definite variation of the intrinsic viscosity with concentration of sodium chloride. The data of Jordan and Porter are given in *Figure 44*. These results thus indicate that the nucleate ion does vary slightly in size with electrolyte concentration and thus possesses some flexibility. Jordan and Porter[85] have calculated, from the values of the intrinsic viscosity of the nucleate ion in solutions of 4×10^{-4}M and 10^{-1}M sodium chloride, the ratio of the lengths of the nucleate ion in these two solvents assuming a random coil model using the Flory–Fox[68] equation and a rigid rod model employing Simha's[64] equation. The results are given in *Table 16* and show that the ion contracts to about three-quarters of its length on increasing the concentration of sodium chloride from 4×10^{-4}M to 10^{-1}M. These results are in conformity with those obtained from light scattering measurements by Geiduschek[86] and by Rowen[45].

Table 16. The ratio of the lengths of the deoxypentose nucleate ion in different solutions[85]

Method of calculation	*Concentrations from which ratio is calculated*	*Ratio*
Flory–Fox (random coil)	4×10^{-4}M and 10^{-1}M NaCl	0·82
Simha (rigid rod)	4×10^{-4}M and 10^{-1}M NaCl	0·72
Light scattering[45]	0 and 2×10^{-1}M NaCl	0·66

Thus Rowen observed that the maximum dimension decreased from 6800 Å to 4500 Å when the sodium chloride concentration was decreased from 0 to 2×10^{-2}M and Geiduschek found that the maximum dimension increased by about 60 per cent when the sodium chloride concentration was reduced from 0·2M to zero. A similar change of solvent produces a tenfold change in the length of the typical polyelectrolyte, sodium polymethacrylate[87, 88]. Although measurements of light scattering at low ionic strengths are subject to error in view of the fact that the inter-ionic repulsions produce a certain degree of order in the solution[52], nevertheless there is sufficient agreement between the methods of measurement to conclude that the nucleate ion is to a slight extent deformable.

Further information on the flexibility of the deoxypentose nucleate ion is obtained from streaming birefringence studies[32, 69, 70, 89–92].

Schwander and Cerf[69] observed that the streaming birefringence decreases with ionic strength although the rotational diffusion constant remains constant. This result was interpreted by Sadron[75] in terms of a rigid rod model. The experiments of Schwander and Cerf[90] in which was measured the birefringence of solutions of sodium deoxypentose nucleate in which the viscosity was varied by the addition of glycerol are probably of greater significance. The birefringence may arise either from the orientation of a rigid particle in the stream lines of the moving solution or from the deformation of a flexible random coil to give increased asymmetry followed by orientation in the stream lines, or by a combination of these two effects. The contribution of these two effects to the birefringence may be determined by varying the viscosity of the solvent[93]. On increasing the viscosity of the solvent, tan α (where α is the initial slope of the curve relating extinction angle and velocity gradient) should be a linear function of the viscosity of the solvent and for rigid rods the straight line relating tan α and the viscosity should pass through the origin; for flexible particles the curve has a positive intercept. The results of Schwander and Cerf[90] (*Figure 45*) and of Horn, Leray, Pouyet and Sadron[94] were first interpreted[75, 90] as indicating that the nucleate ion behaves in solution as a rigid rod, the deviation from linearity at high solvent viscosities being

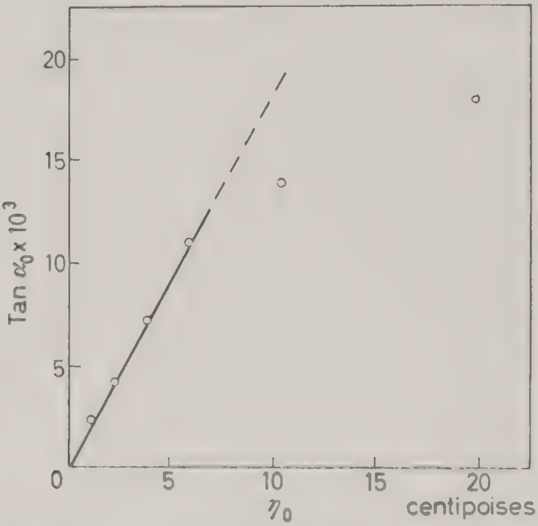

Figure 45. Values of tan α_0 for a solution of sodium deoxypentose nucleate in which the viscosity has been varied by the addition of glycerine

(H. Schwander and R. Cerf: *Helv. chim. Acta* 1951, 31, p. 441) attributed to heating effects. However, Cerf[93] has re-interpreted these results, on the basis of a more extensive theoretical treatment, as indicating that the deviation is a real effect produced by the deformation of the nucleate ion.

From the above discussion it is to be concluded that the model in best agreement with the experimental observation is that of a stiff coil, which possesses some flexibility and which contracts slightly with increasing ionic strength because of a decrease in the intramolecular ionic repulsion. The influence of acid and alkali on the size and shape of the molecule is more appropriately discussed together with the phenomenon of denaturation (see Chapter 11) but it is worthy of note here that the non-hydrogen bonded random coil obtained on treating native deoxypentose nucleic acid with acid is much more flexible, and behaves in general more like a randomly coiled polyelectrolyte than does the native material. This, taken with the other results given above, confirms the conclusion that the Watson–Crick structure is not significantly modified on solution of the deoxypentose nucleate in electrolyte solution.

THE SIZE, SHAPE, AND HETEROGENEITY OF DEOXYPENTOSE NUCLEIC ACID FROM ELECTRON MICROSCOPE STUDIES

The electron microscope is now capable of clearly resolving small particles down to a size of 10 Å; this is well below the resolution necessary to observe particles of the size of nucleic acids and the method should therefore be of great value as an adjunct to the indirect study of size and shape by physico-chemical studies of solutions. Unfortunately, technical difficulties, of which the most serious is that of distinguishing small organic particles from the substrate on which they rest, has retarded its use. Nevertheless, the recent introduction of new techniques has enabled some significant results to have been obtained on deoxypentose nucleic acid. In the interpretation of these results and in particular of those giving the size, it must be remembered that it is the dry particle that is being observed.

The first electron microscope study of sodium deoxypentose nucleate was made by Scott[95], who obtained pictures of long filaments of the nucleate. The lengths and widths of the filaments varied considerably and it was evident that lateral and longitudinal aggregation of the molecules was occurring. Bayley[96] obtained a slightly higher magnification, but observed a polydisperse system of spheres of approximately 100 Å in diameter. Using a new technique of spraying and freeze drying an aqueous solution on to a collodion filmed copper surface[97], Williams[98] obtained pictures of fibrils of sodium deoxypentose nucleate of 15 Å in diameter. These fibrils were connected to each other indicating the presence of a network gel in the original solution. Williams[99] has also been able to estimate from length measurements that $M_w/M_n = 1{\cdot}6$ which confirms the narrow molecular weight distribution assumed by Doty. A similar fibrillar width has been reported by Rowen, Eden and Kahler[44], who also conclude that the molecule (fibril) is slightly kinked and therefore not inflexible. The electron micrographs of Schuster, Schramm and Zillig[100] show a similar fibrillar network to that observed by Williams.

An important new technique for observing macromolecules with the electron microscope has been introduced by Hall[101, 102], which has been particularly successful with nucleic acids. Freshly cleaved mica was selected as a substrate to support the macromolecules because it is smooth to atomic dimensions and also because it is highly hydrophilic. The solution of sodium deoxypentose nucleate was then sprayed on to the mica surface using a commercial throat vaporizer, shadow-cast with platinum and a film of silicon monoxide deposited vertically to back the platinum. Finally a solution of collodion in amyl acetate was flowed over the surface and dried. The resulting film was scored into squares which floated off as the mica was lowered into a trough of water, from which they were picked up on conventional grids. From the measurements obtained a value for the diameter of the molecule of 25 ± 5 Å was obtained in good agreement with the crystallographic value. In some regions where the sodium deoxypentose nucleate is more concentrated, filaments usually several molecules wide form networks of a gel-like structure.

A more careful study, using the same technique, in which particular care was taken to prevent denaturation during the preparation of the specimen for electron microscopy has been made by Hall and Litt[103]. The diameter of the molecules was confirmed as approximately 20 Å, but of greater significance was the observation of free ends of the molecule. In the native state the ends were square indicating that the double helical structure was maintained to the end of each molecule (*Plate 5(a)*). When, however, the double helix dissociates at the ends, the single polynucleotide chains tend to coil up rather than fray out as two well defined strands.

In *Plate 5(c)* are shown two deoxypentose nucleic acid molecules from the same material as that in *Plate 5(a)*, but the lengths are very much shorter than the average. The chief feature of interest in these particles is the nodules which are interpreted by Hall and Litt as

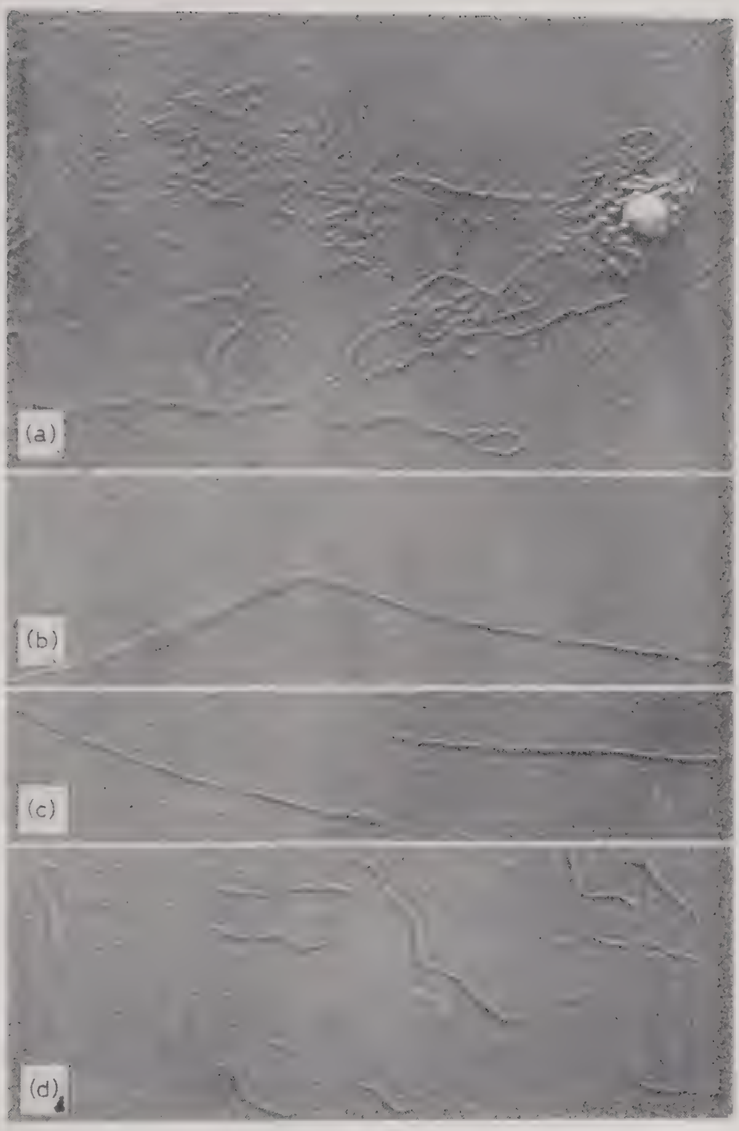

Plate 5. Electron micrographs of sodium deoxypentose nucleic acid of salmon spermatozoa (× *ca.* 100,000)
(Reduced $\frac{2}{3}$ when reproduced)
(*a*) Relatively high concentrations; (*b*) Portions of two branching molecules; (*c*) Two molecules showing thickening of ends associated with partial denaturation; (*d*) Fragmented molecules after sonic irradiation
(C. E. Hall and M. Litt: *J. biophys. biochem. Cytol.* 1958, **4,** Plate 7, figures 1, 2, 3 and 4)

(*To face page 204*)

being the result of partial denaturation of the ends of the molecules. Nodules of this sort are rarely found in the middle of the molecule and when observed are nearly always at the end, which probably indicates that the dissociation of the double helix usually starts at the ends and works inwards along the length. This presents further evidence for a continuous strand model as compared with the interrupted two strand model proposed by Dekker and Schachman[104] (see Chapter 9). In *Plate 5(d)* segments of deoxypentose nucleic acid produced by sonic degradation are shown. The weight average molecular weight as determined from the intrinsic viscosity was 9·7 × 10^5 (approximately a weight average) whereas the number average and weight average molecular weights obtained from the electron micrographs, assuming a mass to length ratio of 200, were 4·2 × 10^5 and 7 × 10^5 respectively[105]. The particles were broken squarely and do not show any of the nodules associated with partial

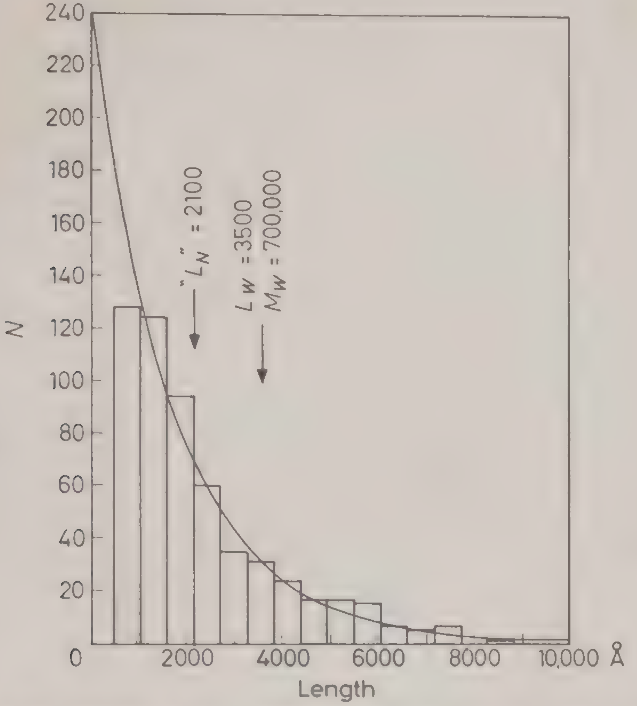

Figure 46. Number and distribution for sonically fragmented sodium deoxyribose nucleate compared with the most probable distribution. Full curve represents most probable distribution; experimental lengths were obtained from electron micrographs.
(C. E. Hall and P. Doty: *J. Amer. chem. Soc.* 1958, **80**, p. 1272)

denaturation. This observation confirms the view of Doty, McGill and Rice[12] that the sonicates are true homologues in which the double helix structure of the native deoxypentose nucleic acid is preserved. When the particles are lying free of each other, they are generally quite straight, but when crowded together tend to be sinuous, thus again confirming the conclusion of the slight flexibility of the molecule.

A study of the distribution of the lengths of native deoxypentose nucleic acid molecules has not been carried out because of the difficulty of measuring the lengths of a number of the molecules, since it is rare to find both ends of an undegraded molecule. Hall and Doty[105], however, have studied the number distribution of a sonically fragmented nucleate and a number distribution based upon length measurement of about seven hundred molecules is shown in *Figure 46*. Particles less than 400 Å in length were not included in the count as it was not always clear whether such small particles were nucleic acid or extraneous material. The weight average length of the distribution was found to be 3500 Å which on the assumption of a mass to length ratio of 200 gives the weight average molecular weight of 7×10^5 referred to above. The number average length was found to be 2100 Å, giving a number average molecular weight of $4{\cdot}2 \times 10^5$. This gives a value for M_w/M_n of 1·7, in good agreement with that obtained by Williams[99] for native nucleic acid.

THE ACID–BASE PROPERTIES OF DEOXYPENTOSE NUCLEIC ACID

The application of electrometric titration of the acid and base groups to the determination of structure has already been discussed in Chapter 9. Here, the determination of the disssociation constants of the acid–base groups at various ionic strengths of both native and denatured deoxypentose nucleic acid will be considered. The nature of the various groups dissociating in a particular pH range may be determined by analogy with the dissociating groups of the nucleotides (see Chapter 7). Primary phosphoric acid groups will have a pK_a' of approximately 1, the amino groups of cytosine, adenine and guanine will titrate in the pH range 2 to 6, and the —NH—CO— groups will dissociate in the range pH 8 to 11.

The most prominent feature of the dissociation behaviour of deoxypentose nucleic acid is that the titration curves, obtained when solutions of sodium deoxypentose nucleate, originally at pH 6 to 7, are first titrated to pH 2·5 or to pH 12, are irreversible. On back-titration from acid or alkaline solution with alkali or acid respectively, the curves obtained differ markedly from the original forward titration (see *Figure 36*, p. 170).

This behaviour was first observed by Gulland, Jordan and Taylor[81] with the sodium deoxyribose nucleate of calf thymus and has since been confirmed for that nucleic acid and for deoxypentose nucleates from other sources[77, 106, 107]. The original titration curves obtained by Gulland, Jordan and Taylor showed exact coincidence of the back-titration curves, that from pH 12 being identical with that from pH 2·5. This coincidence may have been fortuitous since it has not always been confirmed by recent studies using the continuous titration method and similar methods of preparing the nucleate solutions[107, 108]. The lack of coincidence has been ascribed to slight prehydrolysis of the nucleate during the drying procedure[109] (110°C in a vacuum over phosphorus pentoxide[81, 106, 108]) to slow alkaline hydrolysis of the internucleotide linkages during the process of continuous titration above pH 10[107], and to partial reformation of the hydrogen bonded structure on neutralization[63]. Cox and Peacocke[110, 111], however, have carried out a careful study of the sodium deoxypentose nucleate of herring spermatozoa using a non-continuous titration procedure[112] in which a cell without liquid junction is used, and which permits precise control of the ionic strength and also reduces the time at high pH considerably. The results are shown in *Figure 39* (p. 173) where each point represents the results of several determinations on individual mixtures. The points obtained on back-titration after ionization of the amino or —NH—CO— groups lie exactly on the same curve and this is completely reversible. The lack of coincidence of the back-titration curves may thus be regarded as due to an inherent error in the continuous titration method.

The difference between the forward and backward titration curves was interpreted by Gulland, Jordan and Taylor[81] in terms of hydrogen bonds between polynucleotide chains, a view which is in complete conformity with the Watson and Crick structure (see Chapter 9). The coincidence of the back-titration curves shows that there can be no labile linkages connected to the amino and —NH—CO— groups other than the hydrogen bonds between the group on different chains. Furthermore, the titration curves of Cox and Peacocke[110, 111] show that no secondary phosphoric acid dissociations are detectable and the possibility of a broken chain model[81, 104] with terminal secondary phosphoric acid groups, about every twenty nucleotides, is thus excluded.

Studies on the titration behaviour of polymeric acids have shown that as the charge on the polymer changes during titration, due to the ionization of more groups, the effective titration constant of a group alters so that the usual dissociation equation:

$$\mathrm{pH} = \mathrm{pK}' - \log\left(\frac{\alpha}{1 - \alpha}\right)$$

where α is the degree of dissociation and pK′ is the titration constant, has to be modified by the addition of a term dependent on the charge on the nucleate ion and the ionic strength of the solution. Katchalsky, Shavit and Eisenberg[113] have shown that the dissociation of a polymeric electrolyte may be represented by:

$$\mathrm{pH} = \mathrm{pK}_0 - \log\left(\frac{\alpha}{1 - \alpha}\right) + \frac{0{\cdot}4343e\Psi}{kT}$$

where e is the electronic charge, k is the Boltzmann constant, and Ψ is the electrostatic potential. Mathieson and Matty[63], using the values of Ψ obtained by Mathieson and McLaren[114], and given in *Table 17*, have shown that this equation accounts satisfactorily for the departure of the values of the dissociation constant (pK′) from pK_0 at finite concentrations and ionic strengths (see below). Furthermore, the variation of the electrostatic potential (Ψ) with pH is very slight; for example, at $I = 0{\cdot}10$, the value of the term $0{\cdot}4343e\Psi/kT$ increases by only 0·34 units (of pH) from pH 3 to 6 (see *Table 17*). This infers that this term is of no great significance in the case of deoxypentose nucleic acids and that there should be very little spreading of the titration curves of the nucleic acid compared with those of free nucleotides or nucleosides, in marked contrast to the titration behaviour of synthetic polyelectrolytes when compared with that of the appropriate monomers. This behaviour of deoxypentose nucleic acids has been attributed by Peacocke and Lifson[115] to the dominating effect of the primary phosphoric acid groups which at pH values greater than about 2·5 will be fully charged, and to the screening action of the free ions on the potential of the dissociating groups.

Table 17. The electrostatic potential of the deoxyribose nucleate ion ($I = 0{\cdot}10$)

	pH					
	3·0	3·5	4·5	5·0	6·0	7·0
$\Psi \times 10^4$	2·23	2·35	2·47	2·56	2·90	3·20
$0{\cdot}4343e\Psi/kT$	1·14	1·20	1·26	1·30	1·48	1·63

(A. R. Mathieson and J. V. McLaren: *J. chem. Soc.* 1956, p. 306)

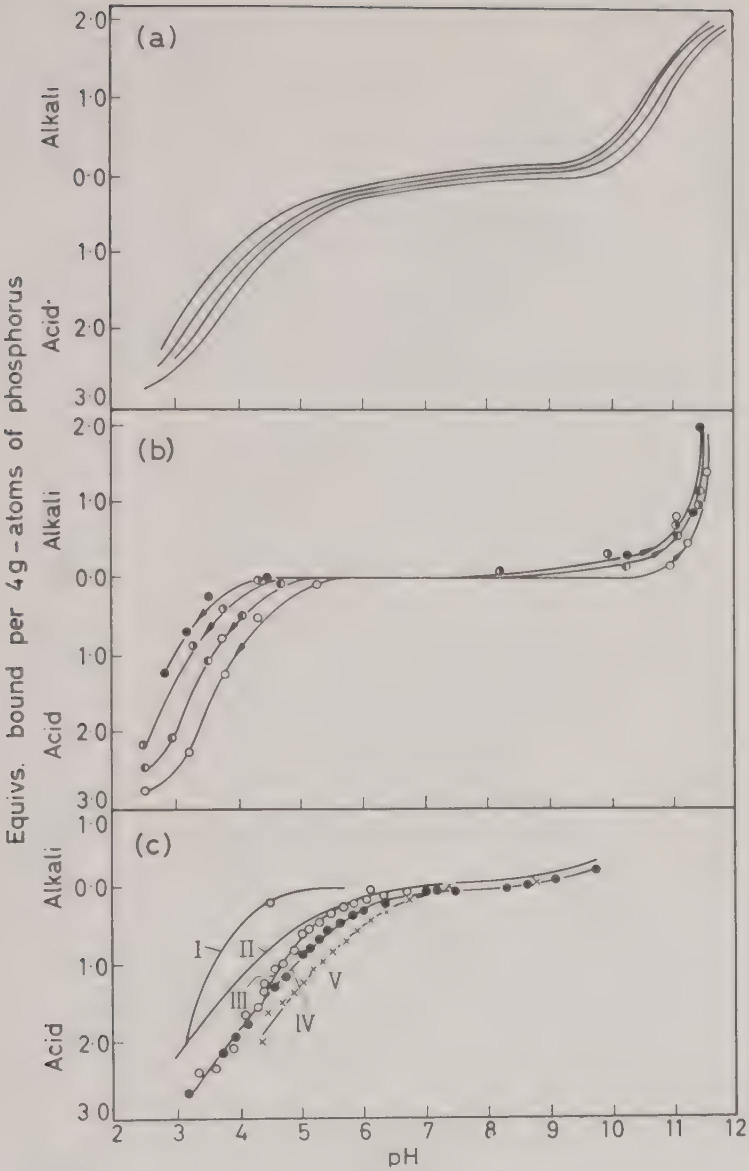

Figure 47. The influence of ionic strength on the irreversible forward titration curves and the reversible backward titration curves of sodium deoxypentose nucleate

(*a*) Reversible back-titration curves, (*b*) Forward titration curves at various ionic strengths ($I = 0{\cdot}02$, 0·05, 0·15, 0·50 reading from left to right), (*c*) Acid titration curves at low ionic strength: I and II, forward and back-titration curves to and from pH 2·5 ($I = 0{\cdot}05$); III and IV, forward (○) and backward (●) titration curves to and from pH 2·5 with no electrolyte added ($I < 0{\cdot}007$); V, reversible titration curve (×) with acid of deoxypentose nucleate previously acid treated, neutralized and dialysed

(R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1956, p. 2503)

The influence of ionic strength on the irreversible forward titration curves and the reversible backward titration curves is shown in *Figure 47*. The reversible back-titration curves are displaced in a parallel manner towards lower pH values with increasing ionic strength, an observation which further substantiates the use of the simple form of the dissociation equation when interpreting them quantitatively. A similar parallel shift has been observed for the effect of ionic strength on the spectrophotometric titration[116]. The parallel displacement of the nucleate titration curves with increasing ionic strength could be caused by the binding of the sodium ions which replace hydrogen ions which are dissociated[110]. This view is substantiated by the very small change in the net charge of the deoxypentose nucleate ion when the ionic strength is changed at constant pH. For example, at pH 6·0, the charge fraction changes from 0·20 (electronic charge per atom of phosphorus) at an ionic strength of 0·02 to 0·22 at an ionic strength of 0·20[114] (see *Table 21*, p. 220).

An alternative explanation of the salt effect has been examined by Cox and Peacocke[110]; this is based on the assumption that the sodium ions are completely dissociated from the nucleate ion and that increasing the sodium chloride concentration causes greater shielding of the ionic atmosphere around the nucleate anion. Using the equation of Hill[117] for the electrostatic free energy of the deoxypentose nucleate ion, the pH change associated with a given change in ionic strength may be calculated. However, it was found that such an explanation would demand a much greater extension of the molecule on ionization than is observed. The first explanation given above, *viz.* that the sodium ions displace the hydrogen ions and become bound to the nucleate, is thus to be preferred and furthermore is in agreement with other, chiefly spectrophotometric, evidence, which indicates that the binding of cations occurs in electrolyte solutions.

The variation of the electrometric titration curves and the titration constants of the acid–base groups with ionic strength has been studied in detail by Jordan, Mathieson and Matty[108, 118] and a similar, but less detailed, study of the influence of ionic strength on the spectrophotometric titration curve has been given by Cavalieri and Stone[119]. The variations of the amino dissociations with concentration of the deoxypentose nucleate and with ionic strength are given in *Table 18*. Also given in *Table 18* are the pK'_a values of the cytosine and guanine amino groups determined spectrophotometrically[119], and the values of the cytosine amino group and the thymine —NH—CO— group determined by

Hurlen, Laland, Cox and Peacocke[120] from titration of apurinic acid (the name given to the product obtained when the purine bases are removed from deoxypentose nucleic acid by treatment with acid or an acidic ion-exchange resin). For comparison, the

Table 18. Dissociation constants of sodium deoxyribose nucleates at different concentrations and ionic strengths (25°C)

Concentration of nucleate equiv. 4P/l. × 10^4	*Ionic strength*	pK_a'			
		Guanine	*Adenine*	*Cytosine*	—NH—CO—
Sodium deoxyribose nucleate of calf thymus ; electrometric titration[118]					
0	0	3·45	4·25	5·25	—
1·13	—	3·35	4·20	5·25	—
1·88	—	3·35	4·15	5·20	—
2·81	—	2·95	3·90	5·10	—
3·75	—	2·70	3·75	4·95	—
7·50	—	2·50	3·70	4·90	—
11·25	—	2·40	3·65	4·85	—
14·2	0	2·60	4·05	5·25	—
14·2	0·133	1·90	2·90	4·15	—
14·2	0·255	1·70	2·60	4·04	—
14·2	0·368	1·60	2·50	3·95	—
14·2	0·492	1·50	2·50	3·95	—
14·2	0·622	1·40	2·35	3·65	—
14·2	0·748	< 1·4	2·30	3·60	—
14·2	0·871	< 1·4	2·24	3·55	—
Sodium deoxyribose nucleate of calf thymus ; spectrophotometric titration[119]					
—	0·008	3·70	—	5·30	—
Sodium deoxyribose nucleate of herring spermatozoa ; electrometric titration[120]					
—	0·050	2·75*	3·50	5·00	10·65 11·30
Apurinic acid ; electrometric titration[120]					
—	0·05	—	—	5·1	10·1
Deoxyribonucleoside-5′-phosphates[121]					
—	0	2·9	4·4	4·6	9·7† 10·0‡

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* Value deduced from difference between back-titration curves of *Arbacia* spermatozoa and avian tubercle bacillus deoxypentose nucleates[122]

† In deoxyguanosine-5′-phosphate

‡ In thymidylic acid

values of the pK'_a of the acid–base groups of the deoxynucleoside-5′-phosphates determined by Hurst, Marko and Butler[121] are also given.

As will be seen from *Table 18*, the pK'_a values of the dissociating groups vary markedly with concentration of nucleate and with ionic strength. The pK'_a values have been calculated from the titration curves using the simple form of the dissociation equation and employing, where necessary, the appropriate analysis of the nucleic acid for the constituent nucleotides. The contribution of the nucleic acid to the ionic strength of the solutions may be taken as negligible in view of the observation[118] that the dissociation constants of phosphoric acid were unaffected by the presence of nucleic acid.

The value for the pK'_a of cytosine is particularly important for the analysis of the titration curves for the presence of a secondary phosphoric acid dissociation. In this respect the titration of apurinic acid is of particular interest, since, in view of the absence of the purines in this acid, the cytosine dissociation may be examined and the difficulties arising from the overlapping of the dissociation of the adenine amino group avoided[120]. The pK'_a value of guanine has also been obtained by a more direct method than analysis of the nucleate titration curve; the deoxypentose nucleic acids of *Arbacia* spermatozoa and tubercle bacilli differ greatly in guanine content and by subtraction of the curves, a dissociation curve largely corresponding to that of guanine may be obtained[122]. The pK'_a values of the cytosine and guanine amino groups determined in these ways were used by Cox and Peacocke[110] in their careful analysis of the titration curve of sodium deoxypentose nucleic acid which led to the conclusion that the back-titration curves were coincident and that no detectable amount of secondary phosphoric acid dissociation was present.

The values of the dissociation constants of the amino groups of adenine and cytosine obtained from the forward titration curve are identical, both groups having a value of 4·4 at 25°C. The values obtained from the back-titration curves, however, differ both from this value and from each other, being 3·65 for adenine and 4·85 for cytosine (data of Jordan, Mathieson and Matty[108], at zero ionic strength and $11{\cdot}25 \times 10^{-4}$ equivs. 4P/l. of nucleate). These results are in agreement with the hydrogen bonded structure of Watson and Crick (see p. 172) since the hydrogen bonds make adenine and cytosine members of very similar eight membered rings. The similarity of the pK'_a value of guanine in the forward and backward titration (2·50 and 2·40 respectively[108]) does not indicate, as was first suggested, that guanine does not participate in the hydrogen bonding since at lower temperatures (see Chapter 11, p. 255) there is a difference in the pK'_a value. At 25°C, kinetic forces are apparently sufficient to break the hydrogen bond involving the guanine amino group once the hydrogen bond involving the cytosine amino group is ruptured by proton addition. This view is supported by the spectrophotometric titration results of Cavalieri and Stone[119] and is in agreement with the suggestion made first by Pauling and Corey[123] that the guanine amino group participates in the hydrogen bonded structure.

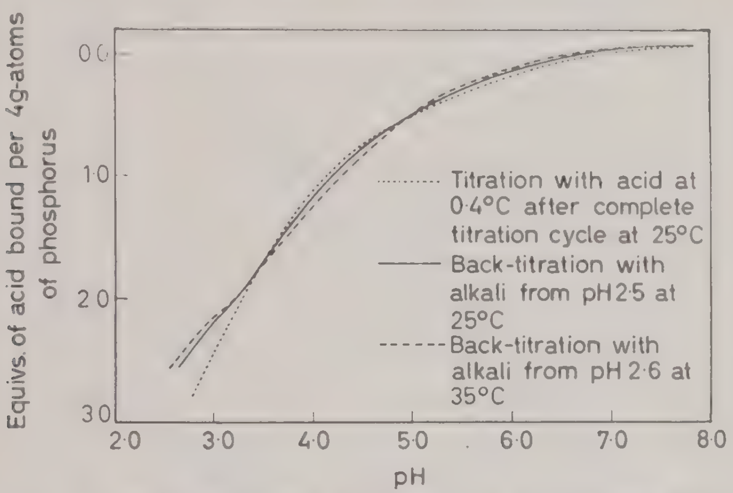

Figure 48. The influence of temperature on the reversible back-titration curve of sodium deoxypentose nucleate
(R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1957, p. 4726)

From a study of the variation with temperature of the electrometric titration curves of the deoxypentose nucleic acid of herring spermatozoa, Cox and Peacocke[124] have been able to determine the apparent heats of dissociation of the amino groups and also to determine the effect of temperature on the reversibility of the forward titration curve. The influence of temperature on the reversible back-titration curves is given in *Figure 48* from which the heats of dissociation have been calculated using the approximate formula:

$$\Delta H' = -2{\cdot}303RT_1T_2\{(\mathrm{pH}_2 - \mathrm{pH}_1)\,/\,(T_2 - T_1)\}_h$$

where h is the number of equivalents of acid or base bound to the nucleate. The values of $\Delta H'$ which are given in *Figure 49* are of the same order as those deduced by Vandendriessche[125] for ribonucleic acid, and it is evident that there is a marked dependence of $\Delta H'$ on h; this has been attributed, following a similar explanation by

Wyman[126] for protein dissociations, to the overlapping of dissociations. Since 80 per cent of a dissociation occurs in the range pH = pK$'_a$ ± 1, then on the basis of the known pK$'_a$ values (*Table 18*) the amino groups of guanine and adenine should overlap below pH 3·75 and those of adenine and cytosine at pH 4·0–4·5. In these ranges of pH therefore $\Delta H'$ should vary with pH, while in the ranges pH 3·75 to 4·0 and 4·5 to 7, where only the adenine and cytosine amino groups respectively are being titrated, $\Delta H'$ should be constant. These predictions are approximately in agreement with the experimental results as shown in *Table 19* (p. 216).

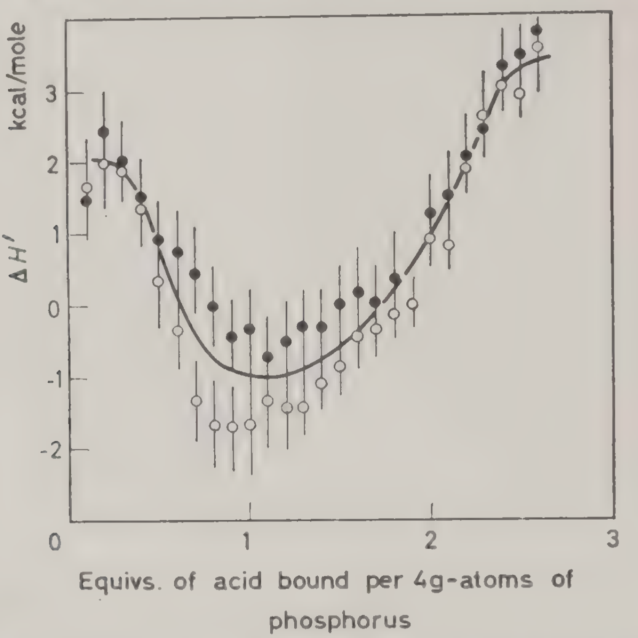

Figure 49. Dependence of the apparent heat of dissociation ($\Delta H'$) of sodium deoxypentose nucleate on the amount of acid bound (h) (R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1957, p. 4727)

The electrometric titration curve of the acid–base groups at 0·4°C is of particular interest in that the back-titration curve obtained after forward titration at the same temperature is neither similar to that obtained at higher temperatures nor coincident with that obtained at 0·4°C after a forward titration at a higher temperature (*Figure 50*, curve II). This back-titration curve lies much closer to the forward titration curve than do those obtained at higher temperatures. On restoring the pH to neutrality it is therefore evident that a large proportion of the original specific hydrogen bonds are reformed and the denaturation process[13] not complete. Cox and Peacocke[124] calculate that 60 per cent of the original hydrogen bonds are retained after having undergone the titration cycle at 0·4°C. These observations which are of particular relevance to the theory of nucleic acid denaturation will be again considered in Chapter 11.

In *Figure 50* the estimated reversible forward titration curve of

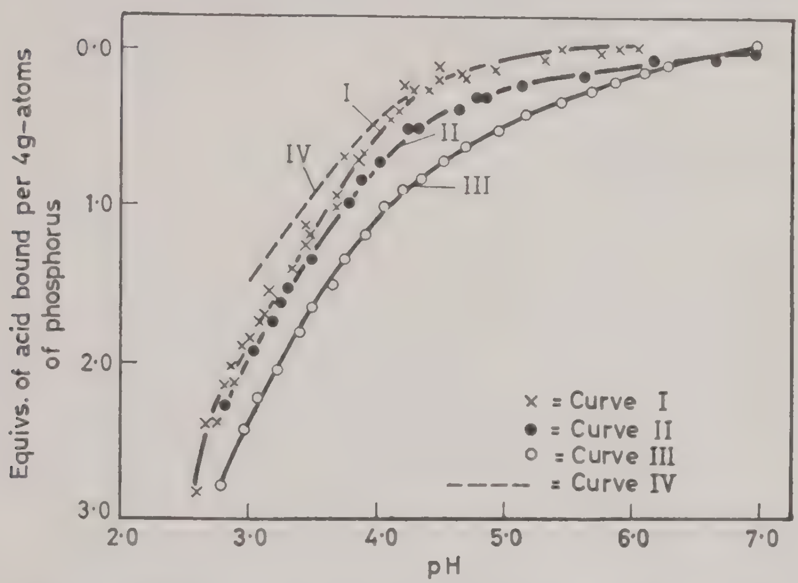

Figure 50. Titration curves of sodium deoxypentose nucleate at 0·4°C in 0·05M sodium chloride

I, forward titration with acid from pH 6·1; II, back-titration with alkali from pH 2·6; III, titration with acid at 0·4°C of nucleate which has previously been titrated at 25°C from neutrality to pH 2·75 and back to pH 7; IV, computed reversible forward titration curve of sodium deoxypentose nucleate in the double helical configuration, *i.e.* without denaturation

(R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1957, p. 4725)

the double helix structure is also given (curve IV). The forward titration curve usually obtained is, of course, complex in nature since the double helix structure is being disrupted during the titration process, so that the last stages of the forward titration process do in fact refer to the denatured nucleic acid. The smaller extent of irreversible denaturation occurring at 0·4°C due to ionization, compared with that obtained at higher temperatures, has enabled an estimate of the titration curve of the hydrogen bonded structure to be made.

Table 19. Interpretation of the variation of $\Delta H'$ with pH

h range	pH *range* (*approx.*)	$\Delta H'$ (kcal/mole)	*Groups assigned*
> 1·40	< 3·75	varying	Guanine-2-amino Adenine-6-amino
1·40–0·85	3·75–4·35	− 1·0	Adenine-6-amino
0·85–0·40	4·35–5·50	varying	Adenine-6-amino Cytosine-6-amino
0·40–0·10	5·50–7·00	*ca.* + 2·0	Cytosine-6-amino
—	9·50–11·0	+ 6·0	Guanine and thymine —NH—CO—

(R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1957, p. 4730)

THE CHARGE ON THE DEOXYPENTOSE NUCLEATE ION

The small variation in shape and flexibility of the deoxypentose nucleate ion brought about by changes in ionic strength, may be related to changes in the charge of the ion. Three methods have been used in measuring this charge. Creeth and Jordan[127] and Shack, Jenkins and Thompsett[128] determined the charge from measurements of membrane potentials. The membrane potential is the potential which exists between a colloidal solution and a buffer solution separated by a membrane permeable to the electrolyte ions, but impermeable to those of the colloid, the two solutions being in Donnan equilibrium. From the membrane potential, the equivalent concentration of the colloidal ion may be determined and then, so long as the molecular weight is known, the charge may be calculated.

The charge on the nucleate ion has also been determined from the electrophoretic mobility. The deoxypentose nucleate ion possesses a high mobility towards the anode[129–133], as would be expected from the dominating effect on the charge of the ionized primary phosphoric acid dissociations. This anodic mobility is dependent on the pH and the ionic strength[114, 133], but almost independent of the nucleate ion concentration[129, 133]. In good preparations, the nucleate migrates as a single boundary which is hypersharp and very similar to that observed in sedimentation. From their measurements of the electrophoretic mobility, Mathieson and co-workers[63, 114] have calculated the net charge at various ionic strengths and pH values using the equation of Gorin[134] which relates the electrophoretic mobility to the charge and the dimensions of the macro-ion. Both these methods of determining the charge are subject to criticism. In the first method it is necessary, in order to obtain reasonable values of the membrane potential, to use high concentrations of the nucleate (*ca.* 0·8 per cent) in which intermolecular interactions will be significant. The charge determined may not therefore bear any significant relationship to that existing at lower dilutions. The second method requires a knowledge of the length and radius of the macro-ion in order to calculate the charge. The values used[63, 114] were obtained from streaming birefringence measurements which, as has been shown above, are not free from criticism. Furthermore, the values of the charge obtained are found to depend markedly on the length taken for the nucleate ion[114].

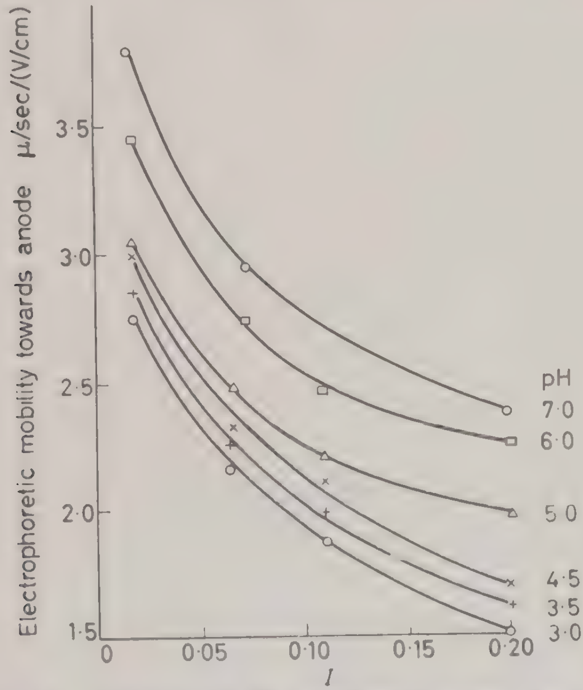

Figure 51. Variation of the electrophoretic mobility of the deoxyribose nucleate ion at various pH values and ionic strengths (*I*)

(A. R. Mathieson and J. V. McLaren: *J. chem. Soc.* 1956, p. 304)

In view of the difficulties associated with the determination of charge by the membrane potential and electrophoretic mobility methods, Inman and Jordan[135] have employed the method of Huizenga, Grieger and Wall[136] which involves determinations of the electrical conductivity and transport. By this method the effective charge fraction on each phosphate group may be determined without any qualifying assumptions. Measurements have been made, however, only on solutions of sodium deoxypentose nucleate free from added electrolyte and the results are therefore complicated by the denaturation process occurring in solutions of low ionic strength (see Chapter 11).

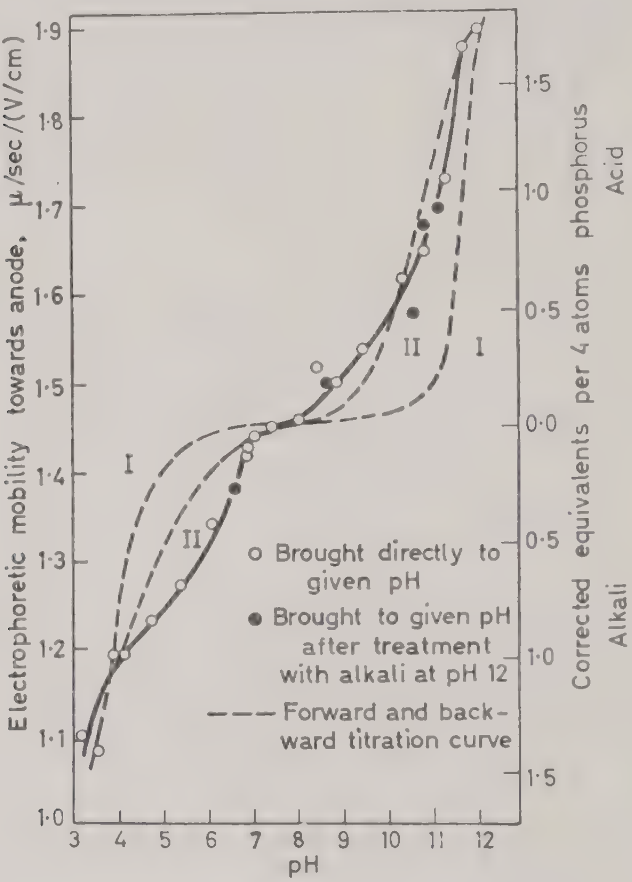

Figure 52. Variation of the electrophoretic mobility of the deoxyribose nucleate ion with pH ($I = 0{\cdot}20$)
(J. M. Creeth, D. O. Jordan and J. M. Gulland: *J. chem. Soc.* 1949, p. 1407)

The variation of electrophoretic mobility with ionic strength and with pH are shown in *Figures 51* and *52*. The sample of deoxyribonucleic acid of calf thymus used by Creeth, Jordan and Gulland[133] differed from that of Mathieson and McLaren[114], the latter being the better preparation. This may be responsible for the somewhat different values of the mobility observed, although the general behaviour is very comparable. The change in mobility with pH may be explained on the basis of the ionization of the acid and base groups. The variation of mobility with ionic strength, however, is more complex and not fully understood (see below) but will be related to changes in the dissociation constants and in the number and nature of the bound gegenions.

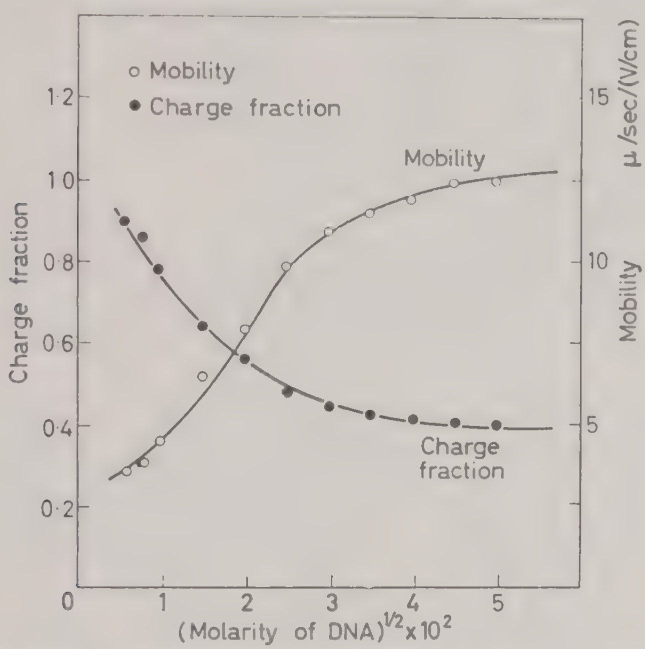

Figure 53. Charge fraction and mobility of the deoxypentose nucleate ion[135]

The values of the charge fraction (*i.e.* the charge per phosphorus atom) on the nucleate ion at pH 7 calculated from membrane potential measurements and from the electrophoretic mobility, making the assumptions described above, are given in *Table 20*. Although there are differences between the values obtained by the various methods, and by different workers using the same method, there is, however, general agreement that the charge fraction is considerably less than unity at pH 7 and in the region of 0·2 to 0·4. Extrapolation of these values to zero ionic strength yields values in the range 0·18 to 0·38 which are in reasonable conformity with the results of Inman and Jordan, given in *Figure 53*, at high nucleate concentrations. At lower concentrations, the charge fractions increase with dilution to give the maximum value of unity at infinite dilution (*Figure 53*).

Table 20. The charge fraction of the deoxypentose nucleate ion at pH 7 obtained by different methods

Ionic strength	*Q*		
	Membrane potential[127]	*Membrane potential*[128]	*Electrophoretic mobility*[114]
0·005	0·21	0·40	—
0·010	0·21	0·45	—
0·020	0·22	0·41	0·21
0·050	0·27	0·52	0·22
0·080	0·29	0·54	—
0·100	0·31	0·53	0·23
0·200	0·37	0·45	0·24

Table 21. The variation of the charge fraction of the deoxypentose nucleate ion with pH and ionic strength

pH	*Ionic strength*			
	0·02	0·05	0·10	0·20
3·0	0·09	0·09	0·09	0·09
4·5	0·17	0·17	0·17	0·17
6·0	0·20	0·21	0·21	0·22
7·0	0·21	0·22	0·23	0·24

(From A. R. Mathieson and J. V. McLaren : *J. chem. Soc.* 1956, p. 305)

Table 22. Theoretical charge fraction on the deoxypentose nucleate ion calculated from pK_a' values

pH	*Nucleate concentration* $\times 10^4$ (*no added electrolyte*)				*Nucleate concentration* $14{\cdot}2 \times 10^{-4}$ *Ionic strength*					
	0	2·81	3·75	11·25	0	0·1	0·2	0·3	0·5	0·7
3·0	0·41	0·43	0·48	0·53	—	—	—	—	0·55	0·61
3·5	0·50	0·55	0·60	0·62	0·50	0·57	0·61	0·67	0·73	0·76
4·0	0·57	0·65	0·70	0·72	0·63	0·70	0·75	0·80	0·86	0·90
4·5	0·74	0·74	0·80	0·81	0·75	0·81	0·86	0·90	0·94	0·96
5·0	0·84	0·86	0·88	0·89	0·78	0·91	0·94	0·96	1·00	—
5·5	0·92	0·93	0·95	0·96	0·93	0·97	0·99	—	—	—
6·0	0·98	0·99	1·00	1·00	0·99	—	—	—	—	—

(Nucleate concentration in equivs. 4P/l.)

(From A. R. Mathieson and S. Matty : *J. Polym. Sci.* 1957, **23**, p. 755)

While at pH 7 there is a small increase of the charge fraction with ionic strength, at lower pH values the charge fraction remains constant within the limits of experimental error[114] (*Table 21*). The difference between the theoretical charge fraction calculated from the titration data[63] (*Table 22*) and the observed charge fraction will be equal to the charge on the bound gegenions. The increase of the negative charge fraction with increase of ionic strength which is shown by all the data given in *Table 20* is unlikely to be caused by a decrease in the amount of bound sodium ion and is more probably due to an increase in the binding of anions (chloride ions)[127]. This view is borne out by the dialysis equilibrium studies of Shack, Jenkins and Thompsett[128] who obtained evidence of chloride ion binding. It would appear reasonable to assume that the sodium ions are only bound by the ionized phosphate groups, since the ionization of these groups is not affected by changes in pH above 2, it is very probable that the number of sodium ions bound remains constant at all ionic strengths[114]. Changes of the charge on the macro-ion with change in ionic strength would then be due to changes in the binding of chloride ions to the amino groups in the ionized or un-ionized form. Whether these ions are strongly bound to the specific groups in the form of ion pairs or more loosely held to the nucleate ion as a result of potential binding cannot be determined from charge determination studies; further consideration of the relative degree of binding of ions will be given in the next section.

THE INTERACTION OF DEOXYPENTOSE NUCLEIC ACID WITH METAL IONS AND WITH ORGANIC CATIONS

The values of the charge fraction on the nucleate ion indicate that a large number of the ionized groups are effectively neutralized by the attendant gegenions which form part of the kinetic unit. The interaction or binding of various ions by the deoxypentose nucleate ion has been studied by observing the change in the ultra-violet absorption spectrum associated with ion interaction, by equilibrium dialysis and by conductimetric titration. The ultra-violet absorption spectrum of deoxypentose nucleic acid shows, in common with other nucleic acids, a well defined maximum at 259 mμ, which is due to absorption by the chromophoric groups of the purines and pyrimidines (*Figure 54*). The extent of the absorption at this wavelength is dependent not only upon the pH and ionic strength of the solution, but also upon the previous treatment of the sample of nucleic acid. Because of the variable water content of nucleic acids, the molar absorptivities cannot be based on the weight of nucleic acid since the ' dry ' weight has little significance; however, nucleic acids contain one phosphorus atom for every purine or pyrimidine and Chargaff and Zamenhof[141] have suggested that the absorption of a nucleic acid solution should be expressed in terms of the absorptivity per gramme-atom of phosphorus per litre. This quantity is now universally used and is given the symbol $\epsilon(P)$.

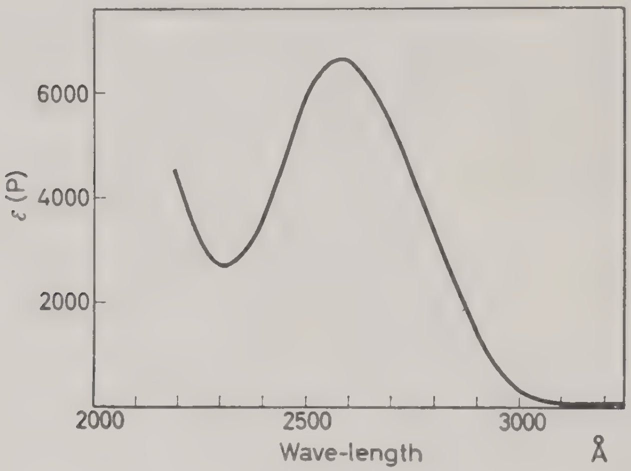

Figure 54. The ultra-violet absorption spectrum of deoxyribose nucleic acid of calf thymus[137]

The value of $\epsilon(P)$ for deoxypentose nucleic acid at 259 mμ under all conditions of pH and ionic strength is very much less than that calculated for an artificial mixture of nucleotides (*Figure* 55[138-140]). On denaturation the value increases, and with further degradation to nucleotides and nucleosides approaches the theoretical value. The absorption will be due to a distinctive electronic axis which is oriented in a particular direction relative to the main axis of the molecule[142] and which is generally assumed to lie in the plane of the purine and pyrimidine ring systems of the Watson and Crick structure, although this has not been proved. Any alteration to the ordered structure either by a rupture of hydrogen bonds or by a breakdown to nucleotides and nucleosides will bring about a more random arrangement of the purine and pyrimidine ring systems. This may produce an increase of $\epsilon(P)$ *per se*[142] or this increase may be caused by an increase in the effective chromophoric area of the ring systems[143]. The interaction between adjacent ring systems in the polynucleotide chains, which occurs even with small synthetic polynucleotides, *e.g.* di-, tri- and tetra-adenylic acid[144] and which has been attributed to dipole interaction[145] and to π-bonding[146] will also be markedly affected by any alteration in the original ordered structure and may be responsible, in part, for the change in absorption.

In view of the sensitivity of the ultra-violet absorption to the orientation of the purine and pyrimidine ring systems in the macromolecule, it is not surprising that the value of $\epsilon(P)$ is very sensitive to changes in structure of deoxypentose nucleic acid. Thomas[145, 147] was the first to observe that solution in pure water or in sodium chloride of concentration less than 10^{-3}M resulted in irreversible changes in structure associated with denaturation. Although these changes will be fully discussed in Chapter 11, it is necessary to emphasize the importance of this observation, as it may affect the conclusions drawn on results obtained prior to the discovery of this effect. In order to prevent the occurrence of this irreversible denaturation it is necessary to dissolve the sodium deoxypentose nucleate in solutions of sodium chloride of concentration greater than 10^{-3}M and in no stage in the preparation of the sample or the solution must the concentration of electrolyte fall

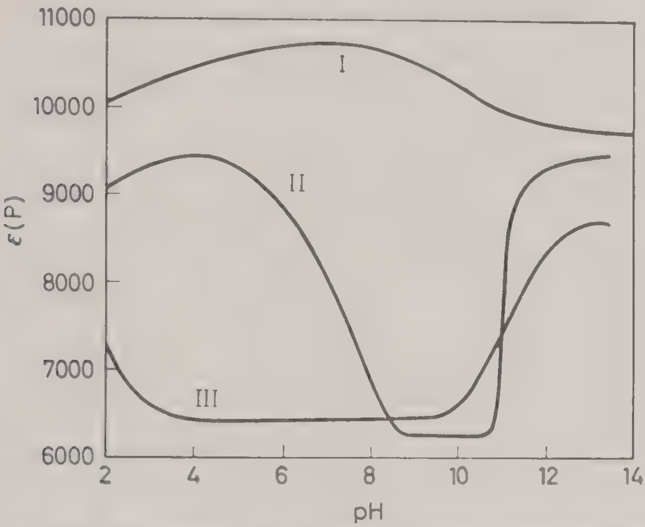

Figure 55. Variation of $\epsilon(P)$ of deoxyribose nucleic acid with pH[138–140]

I, calculated for a mixture of nucleosides containing adenosine, guanosine, cytidine and thymidine in the molar proportion 4 : 3 : 3 : 4; II, variation of $\epsilon(P)$ of deoxypentose nucleic acid in the absence of electrolyte; III, variation of $\epsilon(P)$ for deoxypentose nucleic acid in 1M sodium chloride below this value. In this way solutions with an $\epsilon(P)$ of about 6400 are obtained in 0·1M sodium chloride (*Figure 54*). Exposure of the nucleate to electrolyte solutions of concentration of less than 10^{-3}M brings about an increase of $\epsilon(P)$ to about 7400. The value of $\epsilon(P)$ may thus be taken as a good indication of the state of denaturation of the nucleate. It is, however, possible to dissolve sodium deoxypentose nucleate in water, without apparent denaturation, so long as a concentrated solution is produced; subsequent dilution must then occur with electrolyte solution.

The Binding of Inorganic Ions

The reduction of the $\epsilon(P)$ value of native deoxypentose nucleic acid by the addition of electrolyte was first quantitatively studied by Shack, Jenkins and Thompsett[148] who observed that the optical density of deoxypentose nucleate solutions was lowered by the addition of salts. Although these authors dissolved the sodium deoxypentose nucleate in water in the absence of electrolyte, the values of the maximum at 259 mμ suggest that extensive denaturation had not taken place; however, the results of their studies of the binding of magnesium ions (see below) indicate that the sample has been denatured. The effect of added electrolyte on the absorption at 259 mμ is shown in *Figure 56* where it is seen that the lowering at first increases with the amount of added electrolyte, but at high concentrations no further change takes place. It is evident from *Figure 56* that the effect of added electrolyte is very dependent on the charge of the cation, bivalent cations being effective in lowering the absorption at far lower concentrations than those of univalent cations. The nature of the anion appears to exert little influence. From *Figure 56*, it is also seen that the quantity of any of the bivalent cations needed to bring about the transformation is about that of the quantity of phosphorus in the sodium deoxypentose nucleate. Shack, Jenkins and Thompsett[148] thus concluded that the reduction of $\epsilon(P)$ is associated with the binding of the bivalent cations by the nucleate. This view was substantiated by a study at different nucleate concentrations in which it was found that the extent of the spectral change was primarily a function of the ratio of the bivalent cation concentration to nucleate phosphorus concentration and was not a function of the salt concentration itself. Lawley[140] has obtained similar results and has also attempted to obtain the binding constants of the individual cations by the nucleate; these were found to increase in the order K^+, Na^+, Mg^{2+}, H^+. Cavalieri[149] has also concluded, from a study of the pH changes associated with the addition of magnesium ions to nucleate solutions, that the magnesium ion is strongly bound to the nucleate ion and considers that it is bound both to the phosphate groups and to the amino groups. The evidence for the binding to the latter groups is based on the effect of magnesium ions on the absorption spectrum over the pH range in which the charge on the amino group is changing.

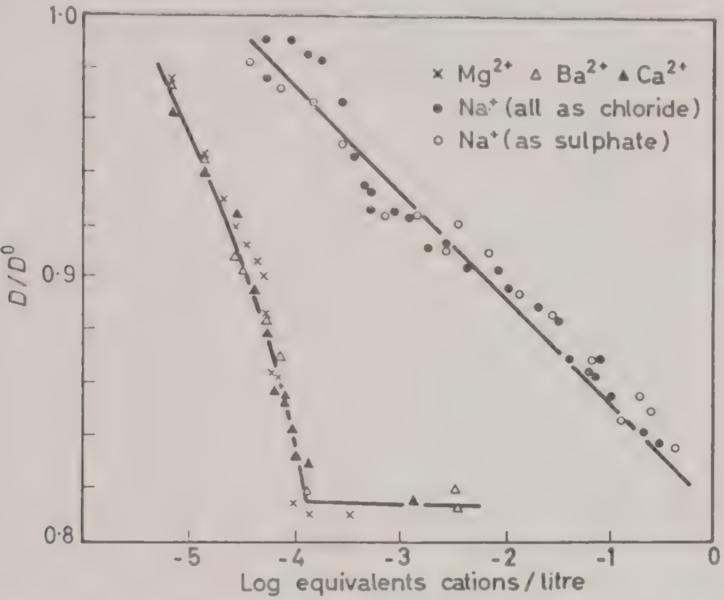

Figure 56. The influence of added electrolyte on the value of the optical density for sodium deoxypentose nucleate
(J. Shack, R. J. Jenkins and J. M. Thompsett: *J. biol. Chem.* 1953, **203**, p. 375)

The interaction of mercuric chloride with sodium deoxypentose nucleate has been studied by Katz[46], but in this case the interaction between the nucleate and the mercuric ion is so great that what is essentially an incomplete mercuric salt of deoxypentose nucleic acid is precipitated. Complexes between deoxypentose nucleic acid and the lanthanides have been studied by Stern and Steinberg[150].

The binding of ions by the nucleate anion may also be studied by comparing the increase of conductivity that occurs when the various electrolytes are added to water and to solutions of sodium deoxypentose nucleate. It would be expected that the latter solutions would show a lower increase compared with that produced by the same concentration of salt in water because of the binding of the cations by the macro-anion. The conductivity behaviour, however, of native deoxypentose nucleic acid is complicated by the marked effect of the addition of salts on the viscosity of the solutions with a consequent increase in the effective mobility of the macro-ion and an increase in the ionic conductance.

Shack, Jenkins and Thompsett[148] first studied the binding of magnesium ions by this method using a sample of deoxypentose nucleic acid which, since it had been dissolved in water in the absence of electrolyte, must now be regarded as having been denatured. For a given concentration of added magnesium chloride it was found that the solution containing the deoxypentose nucleate possessed a lower conductivity than that containing no nucleate. This result was interpreted by Shack, Jenkins and Thompsett[148] as

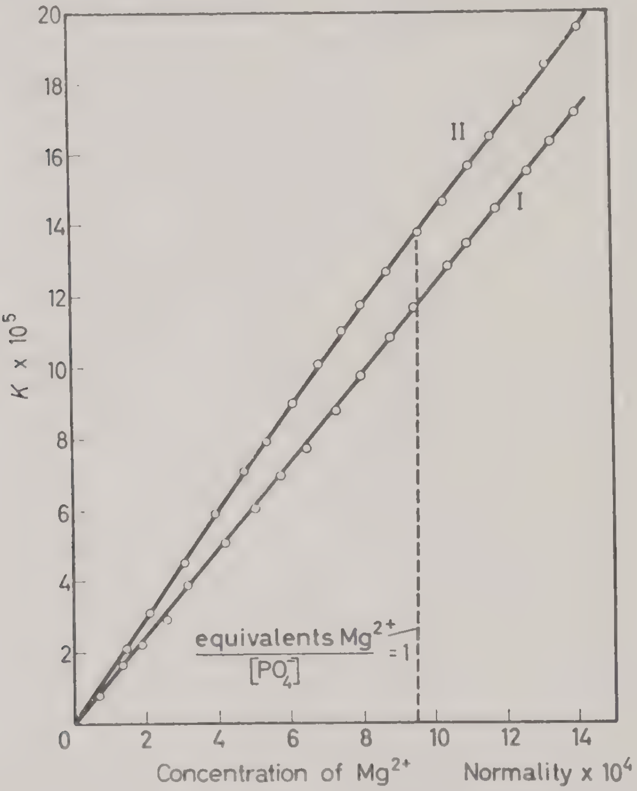

Figure 57. Conductivity titration of sodium deoxyribose nucleate with Mg^{2+}

The increase in specific conductance is plotted as a function of the normality of Mg^{2+}. I, titration of water with Mg^{2+}; II, titration of nucleate with Mg^{2+} (nucleate concentration $3{\cdot}4 \times 10^{-4}$ g/ml.).

(G. Zubay and P. Doty: *Biochim. biophys. Acta* 1958, **29,** p. 51) indicating strong binding of magnesium ions and they calculated that about 0·82 equivalent of magnesium ion was bound to each atom of phosphorus.

Zubay and Doty[151] have repeated this work using a true native sample of sodium deoxypentose nucleate and found, in contrast to the results of Shack, Jenkins and Thompsett[148], that, at a given magnesium chloride concentration, the conductivity of the solution containing nucleate is *greater* than that of the corresponding solution containing water (*Figure 57*). However, a heat denatured specimen of the nucleate showed exactly the same behaviour as observed by Shack, Jenkins and Thompsett in that the conductivity of the solution containing denatured nucleate was lower than that of the same concentration of magnesium chloride in water (*Figure 58*). The explanation of the behaviour of native deoxypentose nucleate given by Zubay and Doty[151] is that the magnesium ions are very effective, in the range of concentration studied, in reducing the specific viscosity of the deoxypentose nucleate solutions as a

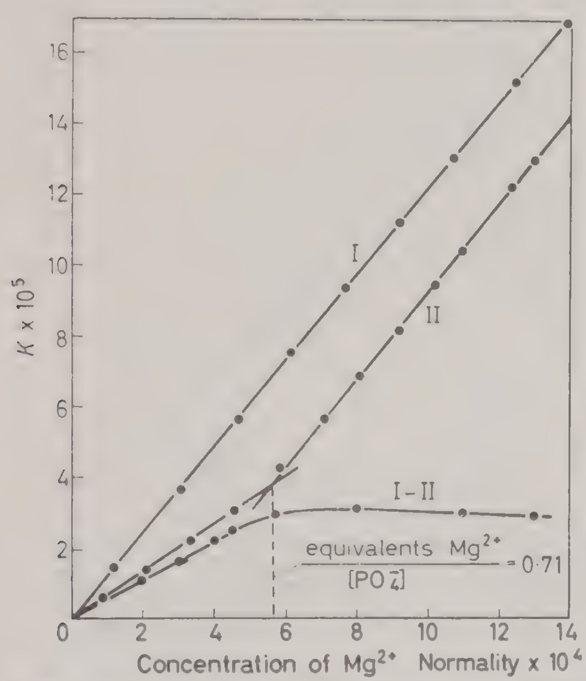

Figure 58. Conductivity titration of denatured deoxypentose nucleate with Mg^{2+}
I, titration of water with Mg^{2+}; II, titration of nucleate with Mg^{2+} (nucleate concentration $2{\cdot}6 \times 10^{-3}$ g/ml.)
(G. Zubay and P. Doty: *Biochim. biophys. Acta* 1958, **29**, p. 52)

result of suppressing the intermolecular interactions. It is considered that the frictional resistance of the nucleate macro-ions is consequently also greatly reduced, a result which would tend to increase the conductivity. This effect presumably more than compensates for any decrease of conductivity caused by the binding of magnesium ions and the over-all conductivity is increased. With cupric ions, however, the degree of binding is such that the conductivity of the nucleate solution is decreased in comparison with the water solution. In this case the lower mobility of the nucleate ion, as a result of binding, more than compensates the suppression of the intermolecular interaction effect which causes the decrease in viscosity.

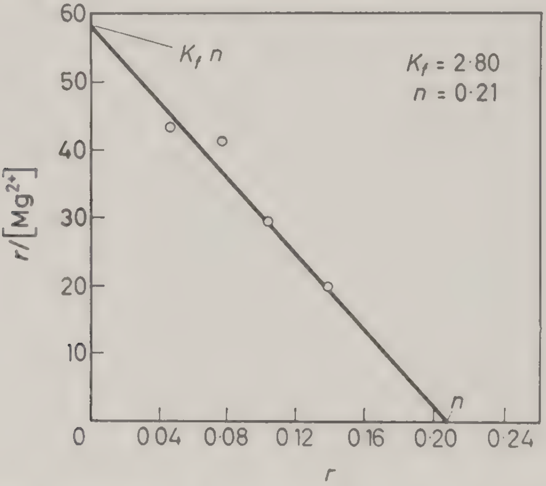

Figure 59. The binding of Mg^{2+} by deoxyribose nucleate in 0·2M sodium chloride. The ratio of Mg^{2+} bound per nucleotide is represented by r; K_f is the association constant and n the number of binding sites per nucleotide.

(From G. Zubay and P. Doty: *Biochim. biophys. Acta* 1958, **29,** p. 50)

In view of the difficulty of interpreting the conductivity results for native deoxypentose nucleate in terms of the binding of magnesium ions, Zubay and Doty[151] have determined this by the method of equilibrium dialysis in 0·2M sodium chloride solution and found the degree of binding (*Figure 59*) very much lower than that observed for the denatured nucleate, for which they obtained the value of 0·71 equivalent of magnesium per phosphorus atom, in reasonable agreement with that of 0·82 given by Shack, Jenkins and Thompsett[148].

The dependence of the equivalent conductivity of solutions of sodium deoxypentose nucleate on concentration gives some information concerning the binding of sodium ions. Most polyelectrolytes are quite flexible molecules and on dilution of their sodium salts only a small increase in equivalent conductivity is observed. This is generally explained as the result of compensation between the greater conductance that would result from dissociation of bound gegenions and the decreased conductance resulting from the expansion of the macro-ion with a consequential increase of frictional resistance. The deoxypentose nucleate anion, however, as has been shown, is relatively inflexible. Consequently when the bound gegenions dissociate from the macro-ion upon dilution the conductivity should increase markedly, because both the number of unbound gegenions and the charge on the nucleate ion increase without a compensating increase in the frictional resistance through swelling. This prediction is in agreement with the equivalent conductivity data (*Figure 60*) and thus it is concluded[151] that the sodium ions are weakly bound by the potential that surrounds the nucleate macro-ion.

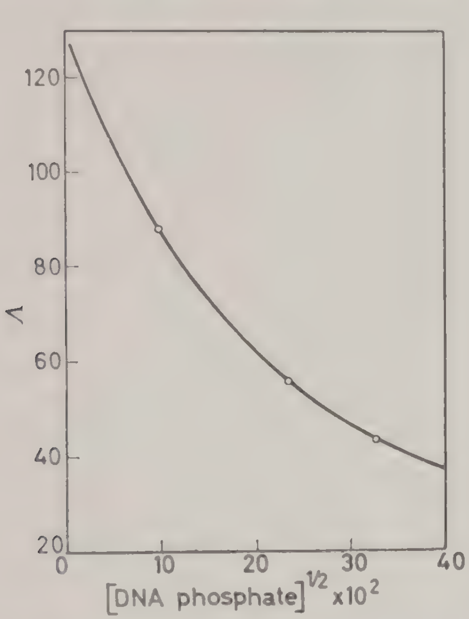

Figure 60. The equivalent conductance of sodium deoxyribose nucleate in water

(G. Zubay and P. Doty: *Biochim. biophys. Acta* 1958, **29**, p. 49)

Zubay and Doty[151] thus conclude that the order of binding sodium, magnesium and cupric ions increases in the series Na^+,

Mg^{2+}, Cu^{2+}. The sodium ions are weakly bound and not located on specific sites, the magnesium ions are bound more tightly but here also it is considered that the degree of binding is not such as would indicate the location of the ions on specific sites. Cupric ions, however, are considered to be tightly bound on specific sites, aggregation occurring through chelation with phosphate groups of adjacent molecules.

The Binding of Organic Ions

The ability of nucleic acids to bind organic dyes has been known for a long time and forms the basis of many histological methods for examining cell tissue, but only recently has any extensive and systematic attempt been made to study the mechanism of the binding process and the nature of the binding sites involved. These investigations have now been extended to the study of the binding of amino acids and proteins. The purpose of studying the binding of amino acids is to investigate the possible existence of specific binding sites which is a requirement of the template mechanism for deoxypentose nucleic acid replication.

The first studies of the interaction of dyes with deoxypentose nucleic acid were made by Bank and Bungenberg de Jong[152] by observing the particular change in the absorption spectrum of certain dyes on interaction with a colloidal particle, known as the metachromatic effect. With some dyes this effect may be brought about by an increase in concentration of the dye itself; this has led to the view that the effect is related to the aggregation of the dyestuff to form colloidal micelles and those dyes which show the effect without requiring the presence of colloidal particles have been shown to aggregate in solution.

The effect has been extensively studied using deoxypentose nucleic acid as the initiator by Michaelis[153, 154], Lawley[155], Massart, Coussens and Silver[156] and Weissman, Carnes, Rubin and Fisher[157]. If the distance between the relevant binding sites (*i.e.* for cationic dyestuffs, positions of negative charge) of a polyelectrolyte is small, the occupation of neighbouring sites by dyestuff cations, if sterically possible, as for those of planar configuration with their molecular planes approximately perpendicular to the polyelectrolyte surface, will constitute the binding of ‘ dimeric ’ or ‘ polymeric ’ dye species, according to whether two or more adjacent sites are occupied. In the deoxypentose nucleate anion, at all pH values above about 1, there is a negative charge every 3·4 Å due to the charged phosphate group. If there is a dye ion bound to each phosphate group, adjacent ions will be sufficiently close to cause van der Waals’ interaction between them so as to produce changes in the absorption spectrum of the dye. Lawley[155] thus considers that the new maxima which arise in the spectrum of dyes showing this effect (*e.g.* rosaniline) are caused by the polymerization of the dye, as it is bound on to the macro-ion as the dye concentration is increased. Thus the bound cations can be divided into three groups: (*1*) monomers, with sites on each side either unoccupied or occupied by sodium ions, (*2*) ' dimers,' with one neighbouring site occupied by a dye cation, and (*3*) ' polymers,' with both neighbouring sites occupied by dye cations. From these considerations it would appear that the binding sites involved are identical with those which bind simple inorganic ions and in fact the metachromatic effect can be suppressed by considerably increasing the concentration of sodium chloride[153].

The conclusion that the sites for the binding of cationic dyes are the same as those binding inorganic ions is substantiated by the results of Cavalieri and Angelos[158] using rosaniline and the method of equilibrium dialysis and by Cavalieri, Angelos and Balis[159] using rosaniline and trimethyl-(*p*-(*p*-hydroxybenzeneazo)-phenyl)-ammonium chloride and the partition analysis method. The results of Irvin and Irvin[160] with 9-aminoacridine are also in agreement with this conclusion. In agreement with the relative strength of binding of monovalent and divalent cations discussed above, Lawley[161, 162], Cavalieri, Angelos and Balis[159], and Peacocke and Skerrett[163] have observed that divalent cations are much more effective in displacing bound organic cations than are monovalent cations.

From their study of the binding of rosaniline by the deoxypentose nucleate ion, Cavalieri and Angelos[158] found that the simple relationship:

$$r/c = kn - kr$$

where r is the amount of dye bound per mole of nucleate, c is the equilibrium concentration, n the number of binding sites per molecule, and k the intrinsic binding constant, was not obeyed. They concluded that this deviation was not due to electrostatic interactions and interpreted their results in terms of two binding sites of different affinity for the dye molecules and suggested that these sites were monoesterified and diesterified phosphate groups. The proportion of monoesterified group that it was necessary to assume in order to interpret the experimental data was between one-fifteenth and one-eightieth of the total. The existence of such a large proportion of monoesterified phosphate groups would suggest that either the molecule is highly branched or that many breaks occur in the single polynucleotide chains as was suggested by Dekker and Schachman[104].

However, although some of the early electrometric titration results did suggest the presence of some secondary phosphoric acid dissociations such as would occur at a monoesterified phosphate group, the recent work does not support this suggestion (see p. 172) and all the evidence implies that the deoxypentose nucleate ion consists of two continuous, unbranched, polynucleotide chains in each double helix. Heilweil and van Winkle[164], who have extended the studies of Oster[165] on the quenching of the fluorescence of acriflavine by deoxypentose nucleic acid by studying the interaction by partition analysis, have also concluded, in agreement with Cavalieri and Angelos, that the binding sites are heterogeneous and that there are a small number of sites with high intrinsic affinity and a considerably larger number with much lower affinities. On the other hand Irvin and Irvin[160], from a study of the binding of 9-aminoacridine, and Lawley[162], on the basis of the binding of 5-aminoacridine and rosaniline, have reported no evidence of heterogeneity of binding sites.

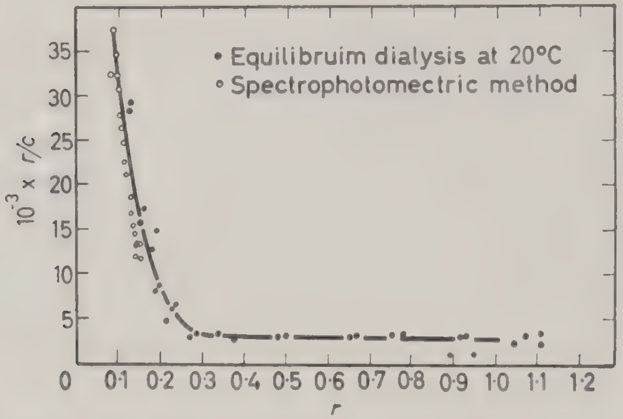

Figure 61. The binding of proflavine by sodium deoxypentose nucleate ($I = 0{\cdot}10$). The number of ligands bound per nucleate at a free ligand concentration of c moles/l. is represented by r. (A. R. Peacocke and J. N. H. Skerrett: *Trans. Faraday Soc.* 1956, **52**, p. 272)

The solution to this problem of the heterogeneity of binding sites appears to have been obtained by Peacocke and Skerrett[163] who, in a very careful study of the binding of various aminoacridines by deoxypentose nucleic acid, did not observe the linear relationship

(*Figure 61*) between r/c and r which is demanded by the simple equation given above. Their results are very similar to those of Cavalieri and Angelos[158] which had been previously interpreted as indicating the presence of two types of binding sites (see above). Peacocke and Skerrett[163], however, have drawn attention to the fact that the binding curve of proflavine on the deoxyribonucleic acid of herring spermatozoa (*Figure 62*) is sigmoidal in shape. This type of absorption curve has been observed previously for the absorption of certain gases on solids and has been interpreted in terms of the interaction of the adsorbed molecules with one another.

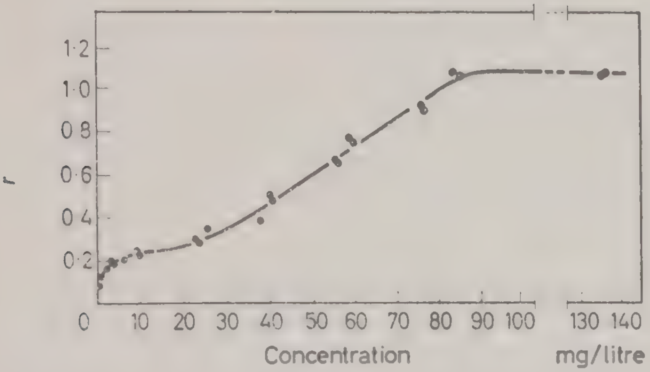

Figure 62. The binding of proflavine by sodium deoxypentose nucleate of herring spermatozoa by equilibrium dialysis at 20°C and pH 6·9 ($I = 0{\cdot}1$). The number of ligands bound per nucleate at a free ligand concentration of c moles/l. is represented by r.

(A. R. Peacocke and J. N. H. Skerrett: *Trans. Faraday Soc.* 1956, **52**, p. 273)

Peacocke and Skerrett have therefore interpreted their results in terms of binding of proflavine molecules and also of proflavine–proflavine interactions on the nucleate surface. This interpretation leads to the conclusion that two distinct binding processes are involved. The first, a strong binding which is predominant when the amount of bound dye is small and involves direct interaction between the dye and the nucleate binding site. The second, a very weak binding occurring when the amount of bound dye is large and involving either the binding of free dye to bound dye or the direct binding of a dye aggregate. It would appear most probable that this mechanism could also adequately explain the results of Cavalieri and Angelos[158] and of Heilweil and van Winkle[164] and it may thus be concluded, in agreement with the general conception of the structure of deoxypentose nucleic acid so far developed, that the binding sites on the nucleate ion are not heterogeneous.

Comparative studies on the strength of binding of different organic cations have been made by Morthland, de Bruyn and Smith[166] and by Lawley[162]. Divalent cations, such as the 2,8-diaminoacridines, are much more strongly bound than are monovalent cations, a behaviour which is analogous to that of simple inorganic cations and is probably due to the simultaneous binding of the bivalent cation to two adjacent phosphate groups. The binding of the aminoacridines is much stronger than that of rosaniline[162] and furthermore the binding is considerably reduced by denaturation of the nucleic acid[162, 163, 167] and by removal of the purine bases to form apurinic acid[163]. This has been attributed to a more specific interaction between the flat ring systems of the purines and pyrimidines of the native nucleic acid and of the aminoacridines probably by π-orbital interaction. Such interaction is additional to the interaction between the charged amino and phosphate groups and will be more specific, varying from one compound to the other.

Experimental evidence for specific interaction between deoxypentose nucleic acid and some amino acids has been sought by Zubay and Doty[151] using dialysis equilibrium methods. No evidence for such specific interaction was observed with serine, glutamic acid or argenine, although the last amino acid was bound more strongly, most probably by non-specific electrostatic interaction, than the other two. The interaction of deoxypentose nucleic acids with proteins has been extensively studied[129, 168–174] by a variety of different techniques and it has been found that under suitable conditions of pH and ionic strength soluble complexes are formed. No evidence of any specific binding has been observed, however, and it has been concluded that the binding process involves no appreciable change in shape of the deoxypentose nucleate ion[173].

STUDIES OF THE PROPERTIES OF DEOXYPENTOSE NUCLEIC ACID IN NON-AQUEOUS SOLVENTS

The only solvent other than water that has been used to study the solvent properties of deoxypentose nucleic acid is ethyl alcohol. Geiduschek and Gray[175] have shown that if aqueous solutions of deoxypentose nucleic acid are first made completely free of electrolyte by prolonged dialysis against distilled water, the nucleic acid may be dissolved in ethyl alcohol by dialysis against gradually increasing concentrations of the alcohol in water, a final dialysis against 98 per cent ethyl alcohol yielding a solution in that solvent.

The work of Geiduschek and Gray has been repeated and extended by Coates and Jordan[176]. The sedimentation boundaries were found to be markedly different from those observed in aqueous 0·2M sodium chloride solution in that they were not hypersharp but spread rapidly yielding a normal symmetrical boundary. The sedimentation coefficients were very high being in the range 50 to 110S although the molecular weight from light scattering measurements[175] showed little change from that observed in aqueous solution. The dimensions of the molecule[175] and the sedimentation and diffusion behaviour[176] indicate that there is a marked apparent decrease in size of the molecule from that of a free draining coil in aqueous solution to one exhibiting non-draining or only partial draining in alcohol solution. This effect, however, will not only be an effect of the change of solvent but will depend on how much denaturation has occurred during the dialysis procedure necessary to bring about the change of solvent.

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11

THE STRUCTURE OF NUCLEIC ACIDS: 4 THE DENATURATION AND DEGRADATION OF DEOXYPENTOSE NUCLEIC ACIDS

INTRODUCTION

In the native deoxypentose nucleic acid molecule, there occur two main types of chemical bonding on which the general features of the macromolecular structure depend. These are the covalent polyester bonds between the nucleotides which form a single polynucleotide chain and the hydrogen bonds between the purine and pyrimidine ring systems of two different polynucleotide chains which produce and stabilize the double helical structure. Decomposition of the native deoxypentose nucleic acid may thus occur by breaking hydrogen bonds, covalent bonds or by rupturing both types of bond simultaneously. By analogy with similar processes occurring in proteins and since, in general, no change in the molecular weight occurs, the breaking of the hydrogen bonds has become known as denaturation[1-3]. This denaturation may be complete if all the hydrogen bonds are broken, but more usually is incomplete, and may under certain conditions be reversible. Rupture of the covalent bonds in the polynucleotide chain, either with or without the accompanying breaking of hydrogen bonds, is a more drastic process which is irreversible and is generally termed degradation. It will be convenient to discuss these two processes for the decomposition of native deoxypentose nucleic acid separately.

THE DENATURATION OF DEOXYPENTOSE NUCLEIC ACIDS

The observation that deoxypentose nucleic acid which had been treated with acid or alkali no longer gave the anomalous titration curve characteristic of the native acid (see Chapter 9) was first made by Gulland, Jordan and Taylor[4]. Ionization of the groups participating in the hydrogen bonding, *viz.* the amino and —NH—CO— dissociations of the purines and pyrimidines, enables some separation of the polynucleotide chains to occur which is only partially restored on standing in solution at pH 7. Subsequently, Cosgrove and Jordan[5-7] showed that heated solutions of deoxypentose nucleic acid likewise did not give the anomalous titration curve. More recently Thomas[3, 8] has shown that irreversible increases in the ultra-violet absorption of solutions of deoxypentose nucleic acid at 259 mμ occur not only on subjecting solutions to heat and extremes of pH, but also on reducing the concentration of extraneous electrolyte below a certain critical value (10^{-3} to 10^{-4}M for sodium chloride, 10^{-5} to 10^{-6}M for magnesium chloride). A similar effect has been shown by Inman and Jordan[9] to occur on diluting a relatively strong solution of deoxypentose nucleate to very low concentrations. Denaturation may also be produced by certain reagents, such as urea, which are known to break hydrogen bonds. It is thus evident that the denaturation of deoxypentose nucleic acid may be brought about by four methods, *viz.* solution in pure water or very dilute electrolyte, subjection to extremely acid or alkaline solutions, heat, and hydrogen bond breaking reagents. These four methods will now be discussed in detail.

Denaturation by Solution in Water or Dilute Electrolyte Solution

The absorption of ultra-violet radiation at 259 mμ by solutions of deoxypentose nucleic acid is very sensitive to changes in structure. As has been discussed in Chapter 10 (p. 222) the value of $\epsilon(P)$ at 259 mμ for native sodium deoxypentose nucleate is much lower than that calculated for a theoretical mixture of nucleotides; denaturation either by heat, acid or alkali has been known for some time to produce an increase in the value. Only extensive degradation, however, by hydrolysis to nucleotides and nucleosides produces a value approaching the theoretical.

Thomas[3, 8] observed that an increase of $\epsilon(P)$ to a value similar to that obtained by heat denaturation occurred on reducing the electrolyte concentration, at constant nucleic acid concentration, of a solution of deoxypentose nucleic acid originally dissolved in relatively strong electrolyte. Furthermore it was observed that this process was irreversible, the addition of electrolyte to a solution of deoxypentose nucleic acid previously dissolved in water or very dilute electrolyte solution, although reducing the value of $\epsilon(P)$, did not reduce it to that of the original native nucleate. This behaviour is illustrated in *Figure 63* where the variation of optical density with changes in concentration of sodium chloride and magnesium sulphate is shown. The original native deoxypentose nucleic acid was dissolved in 1M sodium chloride; on dilution no change in the optical density occurs until a concentration of approximately 10^{-3}M sodium chloride is attained, when the optical density increases to a maximum value, about 25 per cent greater than the initial value, which is reached at about 10^{-4}M sodium chloride. Increase of the sodium chloride concentration to the initial value produces a reduction in the optical density, but to a value about 11 per cent greater than the initial value. The fact that the initial low value of the optical density could not be again obtained led Thomas[3, 8] to the conclusion that an irreversible denaturation had occurred.

The observation that irreversible denaturation of deoxypentose nucleic acid can be brought about by solution of the nucleic acid in pure water or electrolyte concentrations below a certain critical value means that great care must be exercised when extracting deoxypentose nucleic acid from tissues to prevent solution conditions being reached, which would produce a denaturation of this kind. Furthermore the optical density or $\epsilon(P)$ value of a sample of deoxypentose nucleic acid may be taken as an indication of the extent of denaturation that may have occurred by any particular treatment or during preparation. This criterion for a satisfactory preparation of deoxypentose nucleic acid was first suggested by Chargaff[10] (see Chapter 2, p. 14). The minimum values for $\epsilon(P)$ at 259 mμ for a true native preparation, *i.e.* one in which no denaturation has taken place, which have been recorded in the literature, vary from 6000 to 7500, although the majority of values lie in the narrower range of 6300 to 6600. Inman and Jordan[11] from a careful study of five different preparations of the deoxypentose nucleic acid of calf thymus have obtained a value of 6657, the values varying from 6613 to 6705. As stated in Chapter 2 (p. 14) it may be taken that a value for $\epsilon(P)$ at 259 mμ of 6600 refers to a true native preparation.

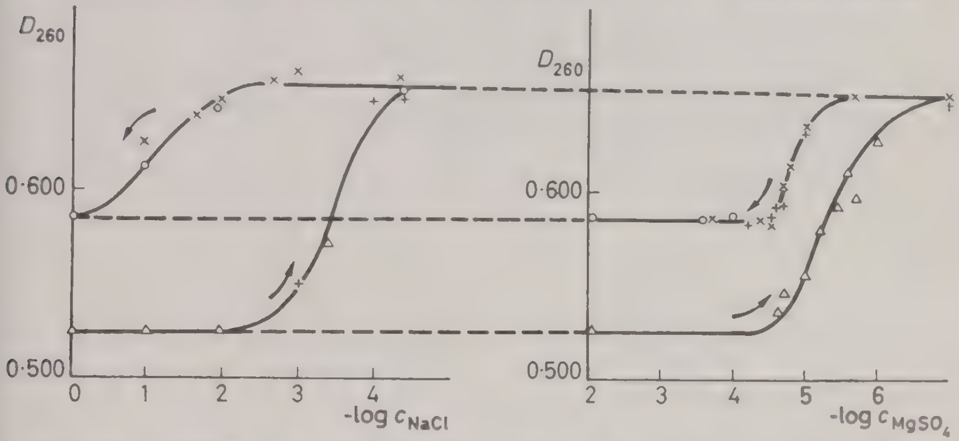


Figure 63. Variation of optical density at 260 mμ of deoxypentose nucleic acid by lowering and raising the concentration of electrolyte
(R. Thomas: *Biochim. biophys. Acta* 1954, **14**, p. 233)

From the data of Thomas[8] given in *Figure 63* it will be evident that the effect of different electrolytes on the optical density of the deoxypentose nucleic acid solutions is similar, the various limiting values obtained by the removal and introduction of electrolyte being the same for sodium chloride and magnesium sulphate. However, with the divalent cation, the limiting concentration at which denaturation occurs is much lower than with the monovalent cation, the respective values being 10^{-3} to 10^{-4}M and $10^{-4\cdot5}$ to 10^{-6}M.

The mechanism of this method of denaturation has been investigated by Cavalieri, Rosoff and Rosenberg[12] whose results confirm and considerably extend those of Thomas. The rise in optical density which occurs on lowering the electrolyte concentration is dependent on the concentration of the sodium deoxypentose nucleate (see *Figure 64*); this is almost certainly due to the

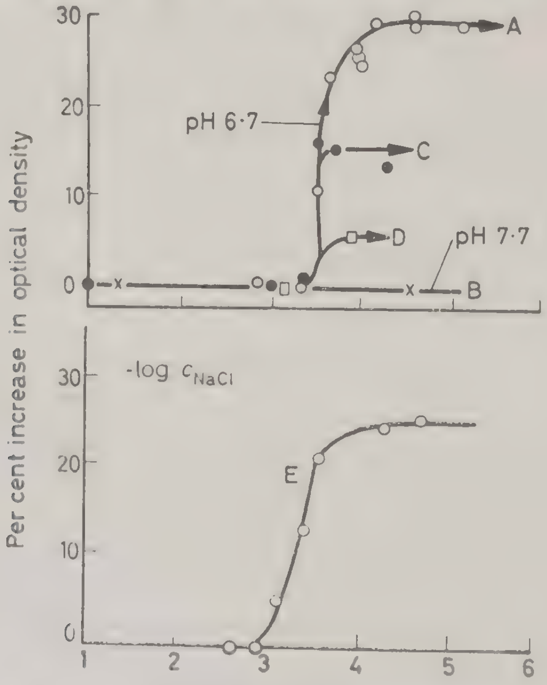


Figure 64. Increase in optical density of sodium deoxyribose nucleate solutions as salt concentration is lowered in neutral solution (pH 6·5–7)

Curves A and B, $0{\cdot}017 \times 10^{-3}$ g/ml.; Curve C, $0{\cdot}12 \times 10^{-3}$ g/ml.; Curve D, $0{\cdot}25 \times 10^{-3}$ g/ml. Curve B shows the effect of decreasing the salt concentration at pH 7·7. In curve E each point represents the optical density increase when a different concentration of nucleate ($0{\cdot}75 \times 10^{-3}$–$0{\cdot}0069 \times 10^{-3}$ g/ml.) is exposed to water plotted against its counter-ion concentration.

(From L. F. Cavalieri, M. Rosoff and B. H. Rosenberg: *J. Amer. chem. Soc.* 1956, **78**, p. 5242) contribution of the nucleate counter-ions to the ionic strength and possibly also to the presence of a small amount of residual sodium chloride in the sodium deoxypentose nucleate due to its precipitation from sodium chloride solution in the preparation. Of fundamental importance to their interpretation of the denaturation mechanism is the observation by Cavalieri, Rosoff and Rosenberg[12] that denaturation does not occur if the sodium deoxypentose nucleate is dissolved in water of pH 8 to 9 so that the pH of the resulting solution is about 7·5 to 8. Although Cavalieri, Rosoff and Rosenberg[12] do not state how this ‘ water ’ was obtained, it was presumably prepared by the addition of a very small quantity of sodium hydroxide to distilled and de-ionized water. Water at pH 7 can be brought to pH 9 by making an approximately 10^{-5}M solution of sodium hydroxide, which is well below the critical concentration required for uni-univalent electrolytes to prevent denaturation, and it was concluded that the absence of denaturation at pH 7·5 was due to the effect of the change of pH and not to the small change in ionic strength. Addition of acid to the solution of nucleate at pH 7·5 produces two sharp spectral rises, one at pH 7 and the other at pH 4·5 (*Figure 65*). Comparison of curves A and B in *Figure 65*, which were obtained in the absence of sodium chloride and in 10^{-3}M sodium chloride respectively, indicate that two different groups [A(1) and A(2)] are involved in the spectral change, one

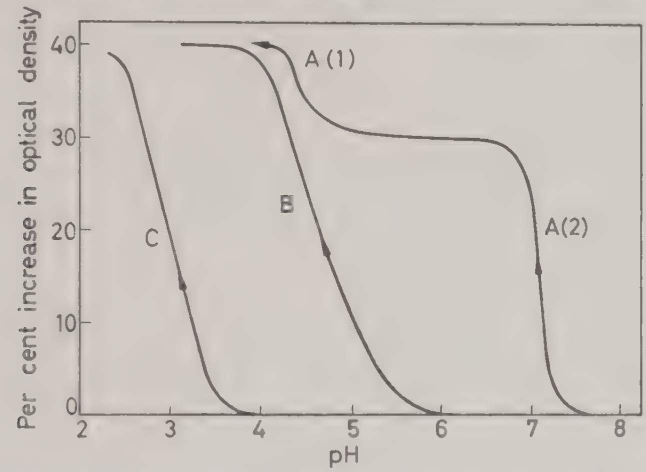
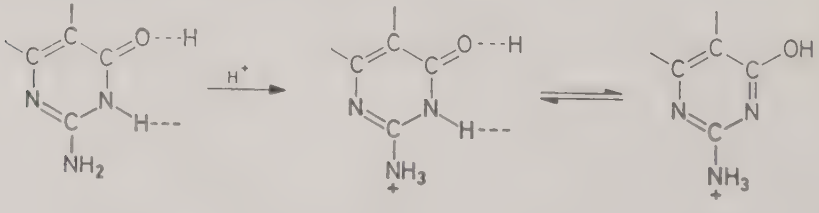


Figure 65. Spectral titration of deoxyribose nucleic acid. The optical density increase is based on undenatured nucleic acid ($\epsilon(P) = 6300$).
Curve A, in water; Curves B and C, in 10^{-3} and 0·2M sodium chloride respectively
(L. F. Cavalieri, M. Rosoff and B. H. Rosenberg: *J. Amer. chem. Soc.* 1956, **78**, p. 5242)

being strongly dependent upon ionic strength [A(2)], the other [A(1)] being little affected by the presence of electrolyte. Cavalieri, Rosoff and Rosenberg[12] have interpreted these results as indicating that the denaturation produced by the removal of electrolyte is, like that produced by acid (see later), the result of proton addition to basic groups in the deoxypentose nucleate. These basic groups may be identified as the amino groups of the purine and pyrimidines which normally have pK_a' values in the range 3 to 5·5 (see *Table 18*, p. 211) but in solutions of low ionic strength the counter-ions which are normally paired to phosphate groups diffuse away leaving unshielded phosphate groups which exert a strong field effect and which, according to Cavalieri, Rosoff and Rosenberg, raise the pK_a' values of the amino groups to about pH 7. Such values are not in agreement with those given in *Table 18* (p. 211) which indicate that the weakest amino group at zero ionic strength is that of cytosine, which then has a pK_a' value of 5·25. However, it must be recalled that these values were obtained by extrapolation to infinite dilution of values obtained at relatively high ionic strengths at which the field effects considered here would not have been operative. It may well be therefore, that the field effect exerted by the unshielded phosphate groups could produce a pK_a' shift of this magnitude.

Cavalieri, Rosoff and Rosenberg[12] interpret the observation that two types of amino group are involved, *viz.* those titrating in the ranges 1 and 2 of curve A in *Figure 65*, by assuming that several deoxypentose nucleate helices are intertwined to form a tight aggregate so that counter-ions bound to phosphate groups in the centre of the aggregate cannot diffuse away readily[13]; these phosphate groups thus remain shielded and cannot exert a field effect on adjacent amino groups which are therefore not subjected to a pK_a' shift. The group which is subject to a pK_a' shift is considered by Cavalieri, Rosoff and Rosenberg[12] to be that of guanine, the shift thus being from pK_a' 2·5 to 7. The mechanism of denaturation is thus pictured by these authors as being proton addition to the guanine amino group with resulting rupture of adjacent hydrogen bonds thus[12]:



(I) Guanine *(II)* *(III)*

However, it is not clear why the guanine amino groups should, on the basis of random aggregation, be the sole groups subject to the field effect, nor is it clear why the final stage in the above denaturation process (*II*)→(*III*) should occur because a proton has been added to the guanine amino group. An alternative explanation could be that the cytosine and thymine amino groups, which take part in the inter-polynucleotide hydrogen bonding, are involved in the proton addition and that the pK_a' values of all the amino groups are shifted by the field effect, which becomes operative at low ionic strengths due to the unshielded phosphate groups. If the pK_a' values of the cytosine and thymine amino groups in the hydrogen bonded structure approach 7 at low ionic strengths, then these would be half neutralized in pure water at pH 7 with the consequent rupture of hydrogen bonds and denaturation of the molecule.

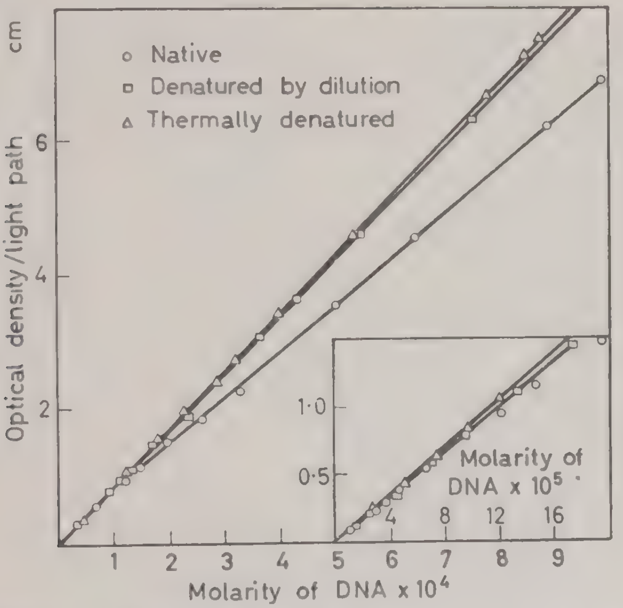


Figure 66. Variation of optical density of sodium deoxypentose nucleate with concentration
(R. B. Inman and D. O. Jordan: *Biochim. biophys. Acta*—in press)

Denaturation of the deoxypentose nucleate may also occur by dilution of the nucleate itself in the absence of any added electrolyte. If sodium deoxypentose nucleate is dissolved in water at reasonably high concentration no denaturation takes place owing to the high counter-ion concentration. As dilution proceeds, the counter-ions bound to the phosphate groups ‘dissociate’ and diffuse away leaving a fully charged phosphate group. Denaturation then proceeds in rather an analogous fashion to that occurring on the removal of electrolyte. This process was observed by Cavalieri, Rosoff and Rosenberg[12], as is evident from the lower graph in *Figure 64* where it is shown that the optical density increases sharply when the counter-ion concentration (and therefore the nucleate concentration) reaches a particular value. This observation was probably first made by Pouyet, Schiebling and Schwander[14] and also by Dhaussy[15] who found a point of inflection in the curve relating optical density with nucleate concentration. This process of denaturation has been studied by Inman and Jordan[9]; points of inflection have been found to occur, not only in the graph relating optical density with concentration (*Figure 66*) but also in that relating electrical conductivity with concentration (*Figure 67*). Both these points of inflection occur in the same range of concentration. Dilution below this critical concentration produces an

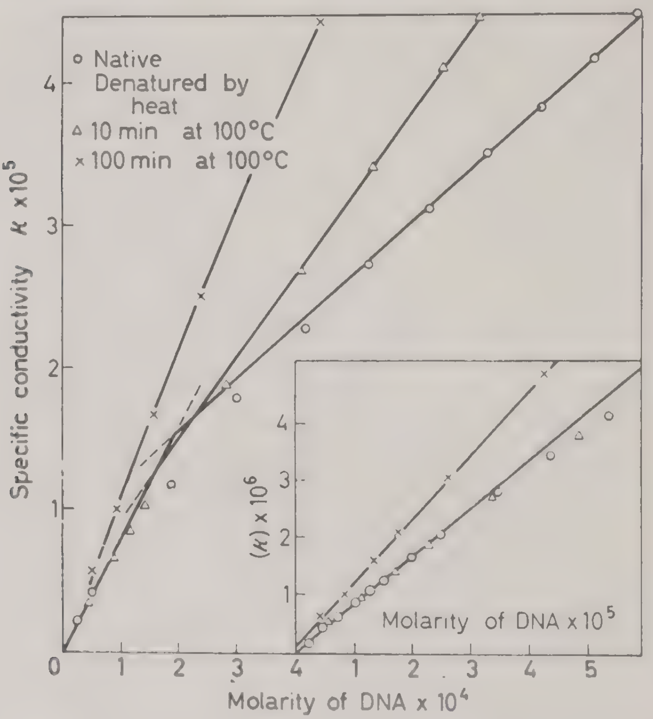


Figure 67. Variation of electrical conductivity of sodium deoxypentose nucleate with concentration
(R. B. Inman and D. O. Jordan: *Biochim. biophys. Acta*—in press)

irreversible change in the deoxypentose nucleate, which is considered by Inman and Jordan to be a denaturation process in view of the similarity of behaviour of the deoxypentose nucleate which has been subjected to concentrations below the critical, with heat-denatured nucleate (*Figures 66* and *67*).

At high dilutions the charge fraction (charge per phosphorus atom) on the deoxypentose nucleate ion increases markedly (*Figure 53*, p. 219) and thus a mechanism based on a shift of the pK'_a values of the amino groups due to the high electrostatic field exerted by the charged phosphate groups, similar to that suggested by Cavalieri, Rosoff and Rosenberg[12] to explain the denaturation on reduction of ionic strength, could be applied. However, rather than considering the denaturation mechanism as involving a proton exchange reaction between the guanine amino group and water, Inman and Jordan prefer to consider the mechanism as a competition by the hydrogen bonded water structure for the hydrogen bonded sites on the nucleate.

Whatever the exact mechanism for the rupture of the specific hydrogen bonds, it is evident from flow dichroism[12, 16] and ultraviolet absorption studies[17] that the denatured molecule lacks the order of the original deoxypentose nucleic acid. This loss of order will result from separation of sections of the two polynucleotide chains following rupture of some of the hydrogen bonds, followed by a rearrangement of the flexible single chains to configurations in which the purine and pyrimidine rings are no longer parallel. Furthermore there is no change in the molecular weight[12] and the root-mean-square end-to-end distance obtained from light scattering measurements does not show any great variation except at high dilution[12]. However, the intrinsic viscosity falls as the degree of denaturation is increased by dilution with water[12] and the molecule becomes more flexible before the optical density commences to rise.

Denaturation by Acid and Alkali

The ability of acid and alkali to bring about irreversible changes in deoxypentose nucleic acid was first observed by Gulland, Jordan and Taylor[4], and by Creeth, Gulland and Jordan[18]. Treatment of deoxypentose nucleic acid at pH 2·5 and 11·5 at 25°C was found to produce a marked change in the titration curve and in the viscosity (see *Figure 36*, p. 170 ; *Figure 37*, p. 171) and the original titration and viscosity properties were not regained on bringing the solution to pH 7. These results were interpreted in terms of a hydrogen bonded structure, the hydrogen bonds joining different polynucleotide chains. It was very relevant to this theory to know whether the acid and alkaline treatment produced any change in the molecular weight, but the early sedimentation and diffusion studies of Cecil and Ogston[19] and Vilbrandt and Tennent[20] were not conclusive on this point. With the formulation and wide acceptance of the Watson and Crick structure for deoxypentose nucleic acid the interpretation of titration, and molecular size and shape studies on a molecular basis became possible and of greater significance.

One of the difficulties of studying the acid and alkaline denaturation of deoxypentose nucleic acid lies in the fact that prolonged treatment with acid and alkali, particularly if the reagents are strong, leads also to degradation of the molecule. It is therefore necessary, if possible, to differentiate experimentally between these two processes. Changing the pH by adding concentrated reagent dropwise to the solution of deoxypentose nucleic acid, as was originally done, must produce some degradation; however, as was first shown by Reichmann, Bunce and Doty[21], the pH may be changed slowly and less drastically by dialysis of the deoxypentose nucleic acid solution against solutions of higher or lower pH. Using this procedure, Reichmann, Bunce and Doty[21], Thomas and Doty[22], Schachman[23], and Coates[24] have studied the size and shape changes accompanying treatment with acid, and Ehrlich and Doty[25] have examined similar changes accompanying treatment with alkali. The light scattering results of Alexander and Stacey[26], while lending some support to the observations of Reichmann, Bunce and Doty, were made on solutions which had been brought to pH 2·2 by direct addition of acid and in the absence of electrolyte. The acid treatment was therefore much less controlled and produced not only denaturation but also degradation.

As the pH is lowered gradually by dialysis in the presence of 0·2M sodium chloride, there is no change in molecular weight down to a pH value of 2·6; there is, however, a marked contraction of the deoxypentose nucleate ion[21, 26]. The values of the radius of gyration calculated from light scattering results were found to be 2050 Å, 1770 Å and 880 Å, at pH values of 6·5, 3·0 and 2·6 respectively[21]. Thus there is a pronounced contraction of the nucleate ion at pH 3·0 which becomes very marked at pH 2·6 where the volume of the ion is approximately one-twelfth that at pH 6·5. These changes in volume calculated from light scattering results are reflected in the changes in viscosity with pH[27–29] (see *Figure 37*, p. 171). The intrinsic viscosity remains at the normal high value of solutions at pH 7 as the pH is decreased until a pH value of about 3·7 is reached when there is a drastic fall between pH 3·7 and 2·8 (Thomas[29] gives the fall as occurring between pH 3·2 and 2·6). Similar results are obtained on exposure of the nucleate ion to alkali[25, 27]. The results of Ehrlich and Doty[25] show that the radius of gyration calculated from light scattering results falls from 2600 Å at pH 7 (ionic strength 0·165) to 950 Å at pH 12, a change in shape and volume which closely parallels that occurring in acid, as shown by the values for the radius of gyration given above. The intrinsic viscosity, similarly, remains at the usual high value observed in neutral solutions as the pH is increased to 12 and then falls sharply[25, 27] (*Figure 37*). Other abrupt changes in the physical properties of solutions of deoxypentose nucleic acid which occur over similar narrow limits of pH in acid and alkaline solution are loss of streaming birefringence[18, 27] (*Figure 38*, p. 171), increase in the ultra-violet absorption at 259 mμ (*Figure 55*, p. 223) and decrease in the optical dichroism[12].

The interpretation most generally accepted for this behaviour is that as the acid or base concentration is increased a point is reached where the acid–base groups participating in the hydrogen-bonded structure undergo proton exchange with the acid or base being added. In the range of pH indicated, however, not all the groups are titrated and as a consequence some hydrogen bonds remain intact. Apart from the regions of intact hydrogen bonds, the polynucleotide chains are free to take up a randomly coiled and flexible configuration with the result that there is a marked over-all contraction of the deoxypentose nucleate ion and an increase in its flexibility[12, 30].

Whether or no the denaturation process is reversible depends upon the proportion of hydrogen bonds that must remain unbroken in order to keep the two polynucleotide chains in register, so that on neutralization the original hydrogen bonds can reform. Furthermore reversibility of the denaturation process does not necessarily infer that the original native deoxypentose nucleic acid has been reformed, as the reformation of hydrogen bonds may occur in a less specific manner on neutralization than occurred in the original native nucleic acid. Information on the reversibility and on the proportion of hydrogen bonds broken in the denaturation process comes largely from electrometric titration studies and from examining the effect of temperature on the acid–base denaturation.

Cox and Peacocke[31, 32] have studied the titration curves obtained by lowering the pH to various selected values on the forward titration curve and back-titrating to neutrality and have thus been able to estimate the proportion of hydrogen bonds which have to be broken in order for the denaturation process to be irreversible.

Lowering the pH of solutions of deoxypentose nucleic acid in 0·05M sodium chloride at 25°C from neutrality, by careful addition of acid with stirring, to various points, *e.g.* E′ and E (*Figure 68*) on the forward titration curve followed by back-titration with alkali gave a family of curves (*Figure 68*) between the forward titration curve APD and the back-titration curve DRA. So long as the addition of acid did not exceed 0·2 equivs. for every 4 g-atoms of phosphorus (*i.e.* titration as far as E′, *Figure 68*) the back-titration curve was coincident with the forward titration curve. This means that at 25°C the ionization of the first 10 per cent of the hydrogen bonded amino groups is reversible, and that so long as a greater proportion of hydrogen bonds are not broken, the deoxypentose nucleic acid may be restored, as far as can be ascertained from titration studies, to its original native form. Ionization of more than this critical proportion of amino groups produces permanent changes in the deoxypentose nucleic acid and titration with acid beyond the point E′ leads to back-titration curves not coincident with the forward titration. Thus forward titration to E (*Figure 68*) leads to the back-titration curve EFA, characteristic of a partially denatured

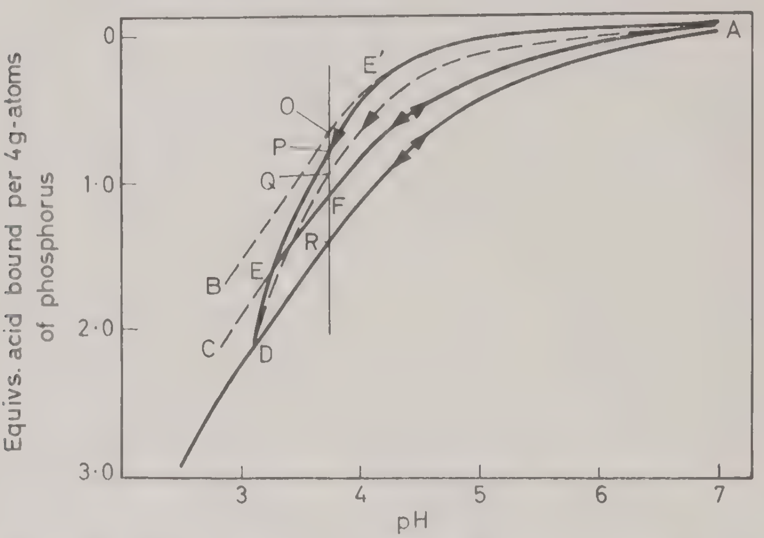


Figure 68. Titration curve of sodium deoxypentose nucleate at 25°C in 0·05M sodium chloride solution
APED, irreversible forward titration curves with acid; EFA, DRA, reversible backward titration curves with alkali from E and D respectively; AOB, reversible titration curve of the hydrogen bonded double helix (*i.e.* undenatured deoxypentose nucleic acid); AQD, forward titration curve for 25 per cent acid denatured nucleate
(R. A. Cox and A. R. Peacocke: *J. Polym. Sci.* 1957, **23**, p. 766)

deoxypentose nucleic acid which is reversible, so long as titration does not proceed beyond E.

At or close to 0°C the forward titration curve with acid of native deoxypentose nucleic acid does not produce the extensive denaturation observed at higher temperatures[31–33] unless the titration is continued to lower pH values[34]. This observation suggested to Cox and Peacocke[31] that a theoretical reversible titration curve of native, hydrogen bonded, deoxypentose nucleic acid may be calculated from a study of the variation of the titration curve with temperature. By titration at 25°C of a sample of deoxypentose nucleic acid which had been taken to pH 3 at 0·4°C and returned to neutrality it was estimated, by comparison with the fully denatured titration curve, that about 60 per cent of the hydrogen bonds remained intact after the titration cycle at 0·4°C. From this result the curve AOB (*Figure 68*), which represents the theoretical reversible titration curve of native deoxypentose nucleic acid, was obtained. Once the reversible titration curves of native and denatured deoxypentose nucleic acid are known (curves AOB and AD respectively in *Figure 68*) it is possible to calculate the fraction (β) of hydrogen bonds irreversibly broken from the position of any intermediate reversible titration curve such as AFE since

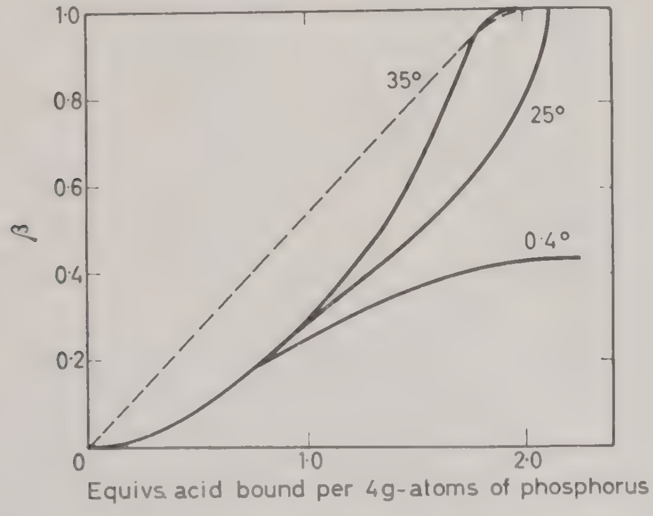


Figure 69. The variation of the fraction of hydrogen bonds irreversibly broken (β) as a function of the amount of acid bound for sodium deoxypentose nucleate at different temperatures; the broken line shows the relation expected if every hydrogen bond was irreversibly broken on denaturation
(R. A. Cox and A. R. Peacocke: *J. Polym. Sci.* 1957, **23**, p. 772)

OF/OR = β where O and R are respectively the points on the native and denatured titration curve at the same pH as any point F on AFE. The variation of the fraction of hydrogen bonds irreversibly broken (β) in the forward titration as a function of the amount of acid bound is given in *Figure 69*, for various temperatures. The broken line shows the relation expected if every group lost its hydrogen bond permanently on denaturation. It is evident that at any given degree of ionization the extent of denaturation is conditioned by the temperature. At 25°C, β is zero at 0·0–0·2 equiv. of acid bound but then increases until at 1·9–2·2 equivs. it increases very steeply to unity. At 35°C, the curve is steeper and complete denaturation ($\beta = 1$) is obtained at 1·86 equivs. At 0·4°C, β does not exceed 0·4 down to 2·2 equivs., while at − 0·75°C, according to Peacocke and Preston[33], β remains at zero.

The titration, with acid, of deoxypentose nucleic acid at various temperatures, is dependent not only on the acid denaturation, but also on heat denaturation. Thus the critical number of hydrogen bonds which must be broken by acid to produce complete denaturation of the molecule is a function of the temperature. This fact was first fully appreciated by Cavalieri and Rosenberg[34], who, like Peacocke and Preston[33], carried out the acid titration at various temperatures. Their results are shown in *Figure 70* and show that there is a common forward and backward titration curve for all temperatures which refer to the reversible titration curves of the native and denatured acid respectively, but that the point on the forward titration curve at which irreversible denaturation commences is dependent on the temperature. These results are not at variance with those of Cox and Peacocke since the points F, F′, *etc.* in *Figure 70* correspond to the point E′ in *Figure 68*, so that before these inflection points the titration curve is reversible, whereas after, it is not. The difference between the reversible forward and backward titration curves, which are independent of temperature, since the temperature coefficient of the pK_a' values is low[34–36], will be due to a shift of the pK_a' values of the amino groups, as suggested by Jordan, Mathieson and Matty[37], the pK_a' value being higher in the hydrogen bonded, native form.

However, the results of Cavalieri and Rosenberg[34] have an important bearing on the interpretation of the titration results. Firstly, the pK_a' values obtained by Jordan, Mathieson and Matty[37] for the hydrogen bonded form of cytosine and adenine will not be correct since they were in fact calculated from a curve of the type AF″D″ (*Figure 70*) in which only the part AF″ is dependent on the pK_a' values of the hydrogen bonded, native amino group, while the part F″D″ refers to a mixture of hydrogen bonded and non-hydrogen bonded groups. Secondly, in view of the interdependence of acid and heat denaturation as shown in *Figure 70*, the conclusion generally reached in the past, that because the forward and backward titration curves are coincident after the addition of about 2 equivs. of acid for every four atoms of phosphorus, the guanine amino group is not involved in the hydrogen bonding, would appear to be invalid (see Chapter 9).

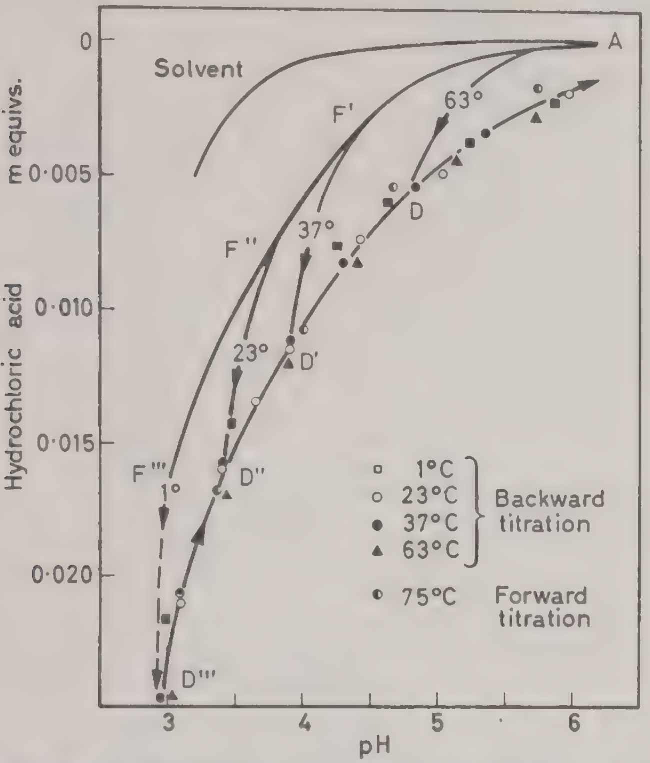


Figure 70. Acid titration curves at various temperatures of sodium deoxyribose nucleic acid. The points indicate curves at each temperature. The forward titration curve at 75°C corresponds with the backward titration curve at lower temperatures. (The broken portion of the forward titration curve at 1°C has been displaced upwards for convenience in plotting). (From L. F. Cavalieri and B. H. Rosenberg: *J. Amer. chem. Soc.* 1957, **79**, p. 5354)

The mechanism of the acid and alkaline denaturation of deoxypentose nucleic acid will clearly involve the addition or subtraction of protons to the acid–base groups participating in the hydrogen bonded structure. This brings about the rupture of the hydrogen bonds and the denaturation of the nucleic acid, although Sturtevant and Geiduschek[36] have suggested, on the assumption that enthalpy changes are proportional to denaturation, that the process of proton addition to the amino group does not necessarily infer hydrogen bond rupture. The suggestion by Cavalieri and Rosenberg[38] and Cavalieri, Rosoff and Rosenberg[12], that the acid denaturation involves the addition of a proton to the guanine amino group with the subsequent rupture of the adjacent hydrogen bonds involving the —NH—CO— group of guanine, must be incorrect in view of the identity of the alkaline and acid denaturation processes as shown by the coincidence of the alkaline and acid back-titration curves. The observations of Cavalieri and Rosenberg[34] that the guanine amino group participates in the hydrogen bonding, as suggested by Pauling and Corey (see Chapter 9) also invalidates this mechanism. The reversibility of the denaturation process will be dependent upon the forces, most probably thermal, tending to disrupt the double helix structure once the hydrogen bonds are broken. If the groups are held in register, even though the hydrogen bonds originally joining them are broken, then the process will be reversible. At 0°C the acid titration is reversible[33] to pH 3 and probably to pH 2·6 and Geiduschek[39] has shown that at this temperature and at pH 2·6 the molecule is nearly as fully extended as in neutral solution. This observation is in marked contrast to that of Reichmann, Bunce and Doty[21] who observed a considerable contraction at this pH at 25°C (see above). It is therefore evident that at 0°C the polynucleotide chains are held in register even though most probably the majority of hydrogen bonds have been broken. Whether this is due purely to a thermal effect, or to the marked increase in the ordered structure of water at 0°C, which Jacobson[40, 41] and collaborators have suggested is affected by the presence of macromolecules such as the deoxypentose nucleate ion, is not clear. In this respect, the observation of Peacocke and co-workers[31–33] that at + 0·4°C titration to pH 2·6 breaks 40 per cent of the hydrogen bonds whereas at − 0·75°C none are broken on titration to this pH, is possibly of significance and suggests that the effect cannot be a purely thermal one. At higher temperatures, as shown by Cavalieri and Rosenberg[34], the pH at which the denaturation occurs increases with temperature.

Denaturation by Heat

Titration studies first demonstrated that an irreversible denaturation occurs when sodium deoxypentose nucleate is heated in neutral aqueous solution[5, 6]. Heat denaturation was later found by Thomas[8] to occur only above a certain critical temperature which is dependent upon the ionic strength as well as on the native state and source of the deoxypentose nucleate. The existence of this critical temperature, which generally lies in the range 70 to 85°C, has

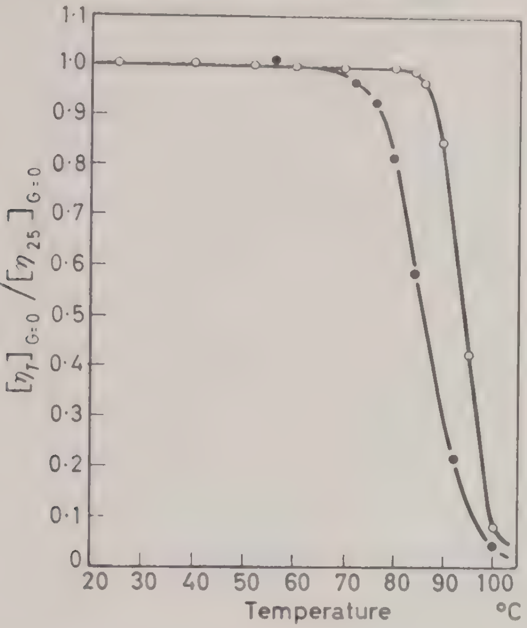


Figure 71. Variation of the intrinsic viscosity of two different samples of sodium deoxyribose nucleic acid with temperature of 1 hour heating

(S. A. Rice and P. Doty: *J. Amer. chem. Soc.* 1957, **79**, p. 3939)

been amply confirmed by a variety of experimental techniques by Goldstein and Stern[42], Zamenhof, Alexander and Leidy[43], Zamenhof, Griboff and Marullo[44], Rice and Doty[45, 46], Hermans and Freund[47], Freund, Pouyet and Sadron[48], and by Cox and Peacocke[49]. The general behaviour is illustrated in *Figure 71* showing the variation of the intrinsic viscosity of solutions of deoxypentose nucleic acid with temperature after 1 hour of heating at that temperature[45]. The viscosity remains constant at the original high value obtained at room temperature until a critical temperature in the range 70° to 85°C is reached, when a sharp fall to a very low value occurs with further increase in temperature. Furthermore, Zamenhof, Alexander and Leidy[43] have shown that the critical temperature at which the fall in intrinsic viscosity occurs is identical with that at which the loss in transforming activity occurs for the deoxypentose nucleic acid possessing transforming activity isolated from *Haemophilus influenzae* (*Figure 72*). This fall of viscosity is not accompanied by any change in molecular weight[45-47] although long periods of heating, especially at or close to 100°C, produce degradation of the molecule with a resulting fall in molecular weight. It is, however, accompanied by a marked decrease in the radius of gyration from the original value for the native nucleate of 2800 Å to an intermediate and essentially constant value at 94°C and a final value of about 1000 Å at 100°C. This contraction represents approximately a fifteen-fold decrease in the mean end-to-end length[46] and is accompanied by a slight broadening in the distribution of sedimentation coefficients without alteration to the mean value.

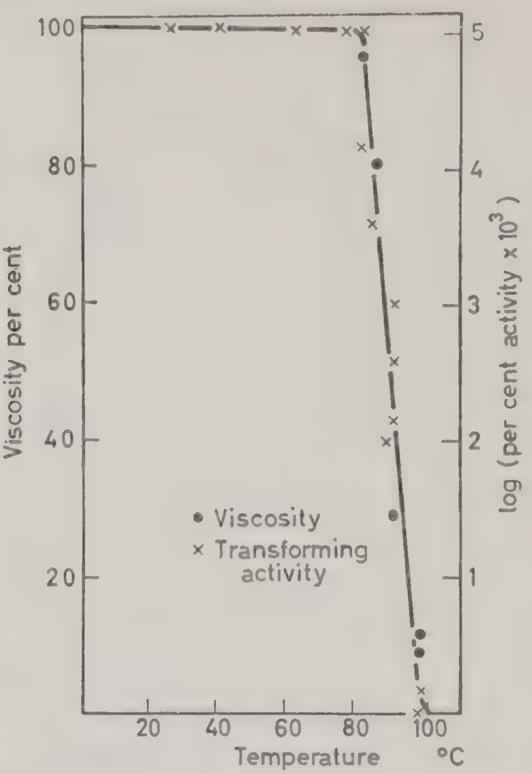


Figure 72. Variation of the viscosity and transforming activity of the deoxypentose nucleic acid isolated from *Haemophilus influenzae*

(The viscosities were all measured at 23°C and the figures given represent the specific viscosities at percentages of the initial viscosity)

(S. Zamenhof, H. E. Alexander and G. Leidy: *J. exp. Med.* 1953, **98**, p. 379)

The sharpness of the fall in viscosity (*Figure 71*) with increase of temperature of heating for 1 hour is indicative of a configurational change having the characteristics of a denaturation of a periodic intramolecular structure and perhaps also of a melting. However, at each temperature above the critical, the viscosity falls with time

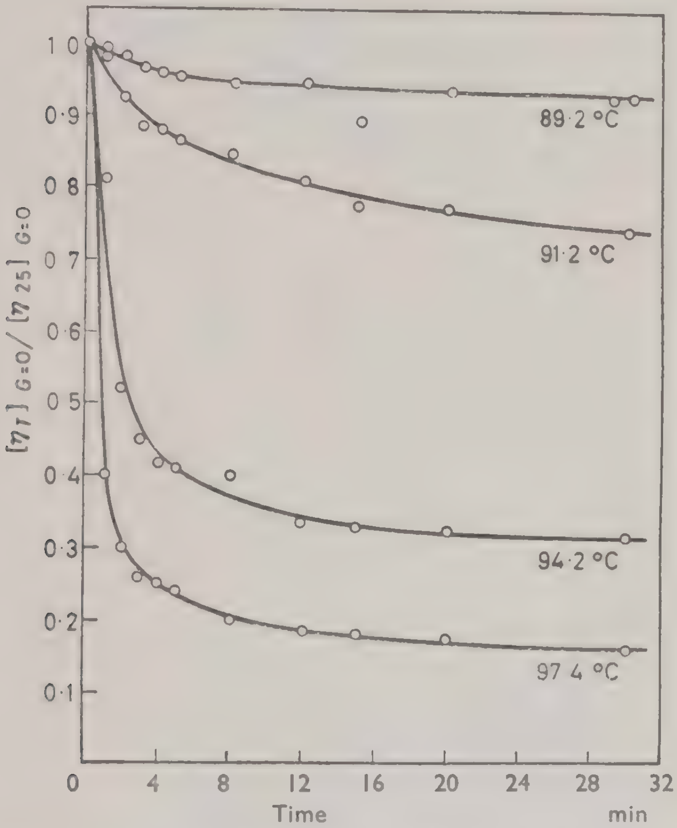


Figure 73. Variation of intrinsic viscosity of sodium deoxyribose nucleate as a function of time at different temperatures
(S. A. Rice and P. Doty: *J. Amer. chem. Soc.* 1957, **79**, p. 3939)

to a level plateau which is characteristic of that particular temperature and not common to all temperatures (*Figure 73*). Thus it appears that the denaturation process proceeds only to an extent which is determined by the temperature. The extent of denaturation at any given temperature may be determined by electrometric titration and Cox and Peacocke[31, 32, 49] have shown (*Figure 74*) that heating for 1 hour at different temperatures produces different titration curves from which difference curves, indicating the extent of hydrogen bond cleavage, may be obtained. The variation of the proportion of hydrogen bonds broken with temperature and calculated in this way shows a critical temperature corresponding to that observed by viscosity measurements and furthermore, closely parallels the rise in optical density with increase in temperature observed by Thomas[8] (*Figure 75*). The step which appears in both these curves would suggest that the denaturation process occurs in two stages, perhaps corresponding to different stabilities of hydrogen bonds.

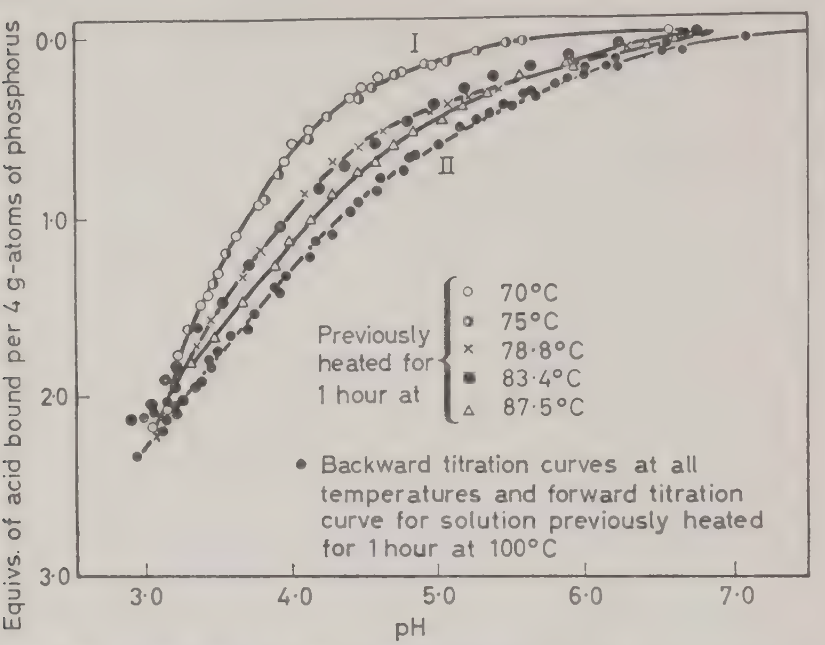


Figure 74. The effect of previous heat treatment on the titration curves of sodium deoxypentose nucleic acid of herring spermatozoa

I and II, forward and backward titration curves respectively of the original unheated nucleic acid. Points shown on forward titration curves are of nucleic acid solutions (0·15 per cent in 0·05M sodium chloride) previously heated 1 hour.

(From R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1956, p. 2648)

It follows also from the observation that the denaturation proceeds only to a limited and characteristic extent at each temperature (*Figure 73*) that the hydrogen bonds are not all of equal strength. This could be related to the known heterogeneity of the deoxypentose nucleic acid molecules and could be interpreted as inferring that some molecules become completely denatured at a given temperature while others are not denatured at all. More probable, however, is the possibility of heterogeneity in the strengths of hydrogen bonds within a given molecule so that a certain sequence of hydrogen bonds in each molecule break at each particular temperature. The over-all compositional heterogeneity will then permit denaturation to proceed further in some molecules than in others. The constancy of the molecular weight throughout the denaturation process indicates that in the fully denatured state the intertwined polynucleotide chains may be held together by some remaining or reformed hydrogen bonds.

From the equilibrium viscosity attained at individual temperatures Rice and Doty[46] have calculated the heat and entropy of reaction, the values being 110 kcal/mole and 300 e.u. respectively. Also from the rate of fall of viscosity with time, the first order rate constants may be obtained and hence the energy and entropy of activation calculated. These were found to be 145 kcal/mole and 325 e.u. respectively. The interpretation of these data is difficult, however, in view of the fact that it is not clear whether they refer to a mole of deoxypentose nucleic acid molecules or to a mole of 'segments' or ultra-molecular regions within the molecules.

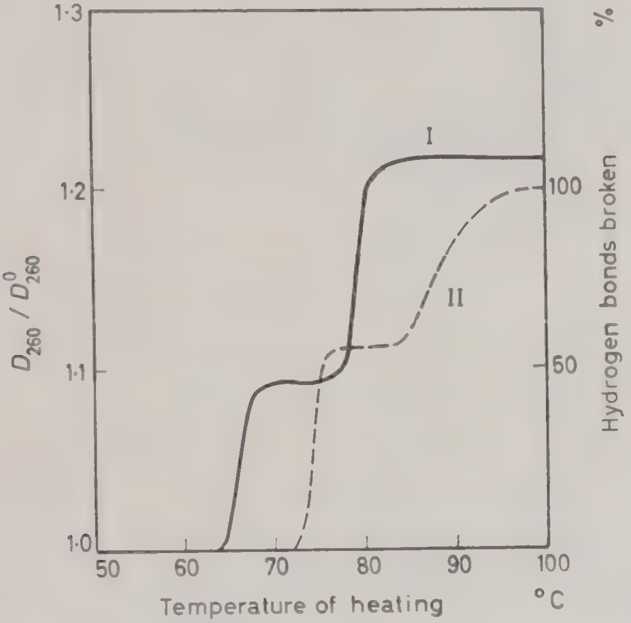


Figure 75. Variation of optical density and the number of hydrogen bonds broken with temperature heating
I, variation of optical density of deoxyribose nucleic acid of calf thymus; II, percentage of hydrogen bonds broken for deoxypentose nucleic acid of herring spermatozoa
(R. Thomas: *Biochim. biophys. Acta* 1954, **14**, p. 235; R. A. Cox and A. R. Peacocke: *J. chem. Soc.* 1956, p. 2651)

The critical temperature at which denaturation occurs is dependent upon the ionic strengths as first shown by Thomas[8] and later confirmed by Zamenhof, Griboff and Marullo[44], and by Litt[50]. Decreasing the ionic strength gives critical values at lower temperatures. This is in conformity with the observations of Miyaji and Price[51] and Sadron[52], which have been confirmed by Coates[24], that the presence of electrolyte exerts a protecting effect on the denaturation of deoxypentose nucleic acid by the action of heat. These observations demonstrate the relationship between the processes of denaturation by removal of electrolyte and by heat.

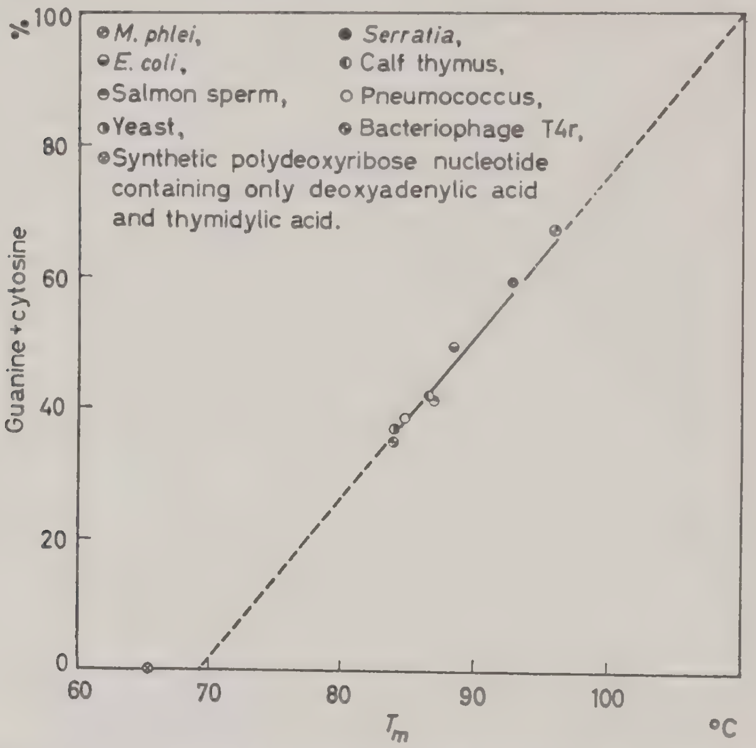


Figure 76. Dependence of the denaturation temperature (T^m) on the guanine–cytosine content of various samples of deoxyribose nucleic acid (J. Marmur and P. Doty: *Nature, Lond.* 1959, **183,** p. 1428)

The critical electrolyte concentration below which, at any given temperature, denaturation occurs, is markedly increased by increase of temperature.

The temperature at which denaturation occurs varies with the source of the deoxypentose nucleic acid as originally observed by Thomas[8]. It may also be related to the heterogeneity of the nucleic acid since Doty, Boedtker, Fresco, Haselkorn and Litt[53], have observed some indication that the transition consists of a series of overlapping transitions of individual nucleic acid molecules. This variation of the denaturation temperature with different molecules could only occur if the hydrogen bonds formed between different base pairs were of different strengths. This has recently been confirmed by Marmur and Doty[54] by relating the denaturation temperature to the constitution of the deoxypentose nucleic acid. In *Figure 76* the denaturation temperature is shown to be a function of the guanine–cytosine content of various nucleic acids which extrapolates to 69°C at zero guanine–cytosine content which is in excellent agreement with the denaturation temperature of a synthetic adenine–thymine deoxyribonucleic acid.

The Action of Denaturing Agents on Deoxypentose Nucleic Acid

Substances such as urea, guanidine hydrochloride and sodium salicylate which are effective in denaturing proteins might be expected to promote the denaturation of deoxypentose nucleic acid.

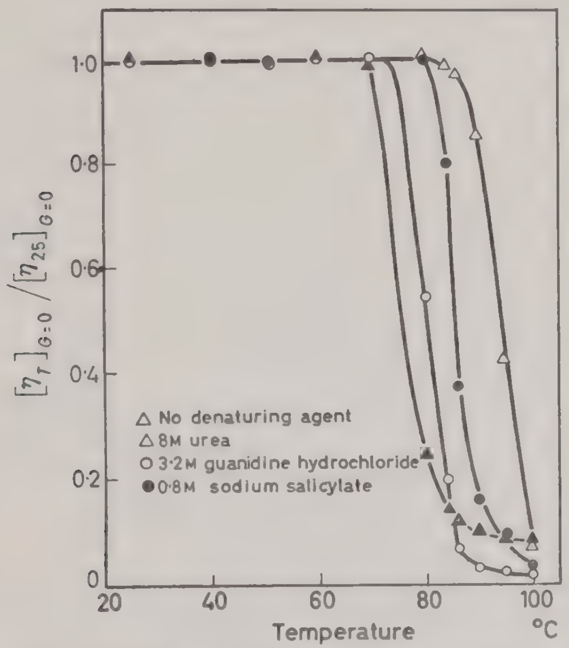


Figure 77a. Variation of intrinsic viscosity of sodium deoxyribose nucleate with temperature of 1 hour heating in the presence of hydrogen bond breaking agents

(S. A. Rice and P. Doty: *J. Amer. chem. Soc.* 1957, **79**, p. 3939)

The effect of 8M urea, 3·2M guanidine hydrochloride and 0·8M sodium salicylate was studied by Rice and Doty[46] who showed that the critical temperature at which heat denaturation occurred was lowered by the presence of these agents (*Figure 77a*). The action of urea in bringing about the denaturation of deoxypentose nucleic acid has been fairly extensively investigated, although the results obtained by various investigators are somewhat at variance. At room temperature Rice and Doty[46] found that urea had no effect on the intrinsic viscosity in 0·15M sodium chloride solution although Conway[55] observed a rise. Conway and Butler[56] observed a decrease in the relative viscosity of deoxypentose nucleic acid on the addition of urea which was not regained on removal of the urea by dialysis. However, these experiments were made before the importance of not reducing the electrolyte concentration below certain limits was appreciated. Mathieson and Porter[57] have found that the addition of urea to solutions of deoxypentose nucleic acid in 0·2M sodium chloride solution increases the flexibility of the molecule as determined by streaming birefringence measurements, which would be in agreement with the cleavage of some of the intramolecular hydrogen bonds.

Alexander and Stacey[26, 58–60] claimed, on the basis of light scattering studies of deoxypentose nucleic acid in 8M urea, that denaturation under these conditions was complete and that the double helix dissociated into two polynucleotide chains each of half the molecular weight of the original molecule. The results have been criticized by Doty and Rice[45] who consider that light scattering measurements cannot be made under the conditions used by Alexander and Stacey. Moreover, in view of the fact that no other method of denaturation, even when shown to be complete by titration studies, leads to a change in molecular weight, these observations would appear to be incorrect.

We may therefore conclude that reagents known to bring about the denaturation of some proteins by disrupting a hydrogen bonded structure, do not by themselves bring about any marked denaturation of deoxypentose nucleic acid, although they can assist in the denaturation produced by other methods.

Conclusions Concerning the Denaturation of Deoxypentose Nucleic Acid

The denaturation studies described above fully confirm the hydrogen bonded structure proposed by Watson and Crick. The biological importance of such studies lies in the relationship between the denaturation and the possible replication mechanism. If the replication of deoxypentose nucleic acid follows a mechanism in which one polynucleotide chain acts as a template for the synthesis of deoxypentose nucleic acid, it will be evident that the specific hydrogen bonds play a vital role in such a process. By following the reverse process, *viz.* the denaturation, we can obtain information concerning the nature of the forces necessary to hold the two polynucleotide chains in register.

The denaturation studies have also led to important conclusions concerning the structure of the double helix. On the basis of degradation studies, both by heat and by the action of enzymes, Dekker and Schachman[61] suggested that the two polynucleotide chains were discontinuous. However, the absence of a change in molecular weight during any of the procedures which lead to denaturation would appear to invalidate this conclusion, since, if the polynucleotide chains were discontinuous, it would appear almost certain that some would separate from the main molecule on rupture of all the hydrogen bonds.

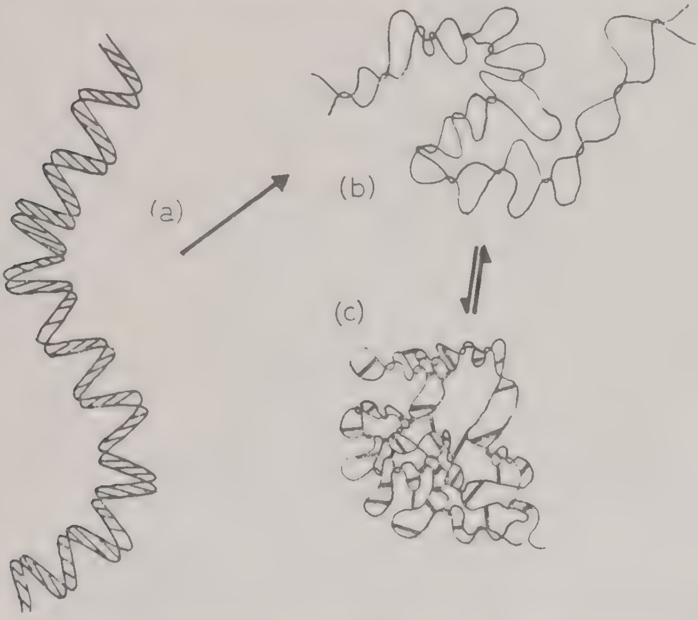


Figure 77b. Diagrammatic representation of the denaturation of deoxypentose nucleic acid

(*a*) represents the undenatured twin helix, which on denaturation yields two intertwined, non-hydrogen bonded polynucleotide chains (*b*). Removal of the denaturing conditions permits the reformation of some non-specific hydrogen bonds. The change from (*a*) to (*b*) is irreversible, while the change from (*b*) to (*c*) is reversible.

(R. B. Inman and D. O. Jordan: *Biochim. biophys. Acta*—in press)

We may thus conclude that the various methods by which denaturation can occur all produce, by rupture of the hydrogen bonds, a collapsed molecule which is more flexible than the native molecule and in which the two polynucleotide strands are intertwined. The consequent loss of order in the arrangement of the purines and pyrimidines results in the increase in ultra-violet absorption and is responsible also for the high entropy change associated with the denaturation process. Diffusion of the two polynucleotide chains away from each other is probably prevented by some residual or reformed hydrogen bonds, or perhaps at low temperatures by alternative hydrogen bonds forming with the water structure (*Figure 77b*).

THE DEGRADATION OF DEOXYPENTOSE NUCLEIC ACID

The prolonged action of some of the reagents that bring about denaturation will produce degradation of the deoxypentose nucleic acid molecule. Thus treatment with strong acid and alkali and heating at 100°C for prolonged periods will bring about first denaturation followed by degradation of the nucleic acid, either by the rupture of phospho-ester bonds or by the removal of purines. Ultrasonic and ionizing radiation will produce degradation, which, under certain conditions, will occur without first disturbing the hydrogen bonded structure. Enzyme depolymerases will also effect the degradation of the molecule.

The action of acid on deoxypentose nucleic acid at about pH 2·6, produces, as has been shown above, denaturation of the molecule without change in molecular weight. Under more strongly acid conditions, purines (adenine and guanine) are liberated and internucleotide bonds are broken. Tamm, Hodes and Chargaff[62] have shown that at pH 1·6 and 37°C all the purines are removed in about 24 hours, by which time the polynucleotide chains have been broken down into small fragments. This degraded product is known as apurinic acid. Further exhaustive hydrolysis will yield the pyrimidines, sugars and phosphoric acid (see Chapter 4, p. 66). The process of acid degradation is thus resolved into the two simultaneous mechanisms, the rupture of phospho-ester and glycosidic bonds. Thomas and Doty[22], from a study of the kinetics of the mild acid hydrolysis of deoxypentose nucleic acid at pH 2·6, have suggested the possible existence of a third type of degradation involving the rupture of residual hydrogen bonds in the collapsed nucleic acid molecule. The rates of these processes differ markedly[63]. About 24 adenine and the same number of guanine molecules are liberated per deoxypentose nucleic acid molecule (weight average) per hour. The rupture of phospho-ester bonds is very much slower being about 0·1 bond per deoxypentose nucleic acid molecule per hour. The residual hydrogen bonds, the number of which is estimated by Thomas and Doty[22] to be about seven per molecule, appear, somewhat surprisingly, to be very resistant to acid action, since according to Thomas and Doty they decay only at a rate such that they have completely disappeared after 50 hours (at 25°C and pH 2·6). The evidence for the existence of these hydrogen bonds is rather circumstantial and is based on the evidence that up to about 50 hours the degradation of the deoxypentose nucleic acid would appear to be that of the double helix, whereas after this time the polynucleotide chains separate and degradation of the single chains occurs. The results of Thomas and Doty[22] lend further support to the rejection of the discontinuous strand model for native deoxypentose nucleic acid proposed by Dekker and Schachman[61], since if these breaks in the polynucleotide chains already existed in the native acid, it would be expected that the molecular weight would decrease much more rapidly than was in fact observed.

The effect of further heating of solutions of sodium deoxypentose nucleate at 100°C beyond that necessary to produce denaturation brings about extensive degradation of the nucleate[46]. The molecular weight, determined from light scattering, is found to remain constant for about 30 minutes during which time the denaturation reaction proceeds to the equilibrium condition. Thereafter the molecular weight falls rapidly to a value approximately one-third of the original in about 2 hours. The nature of the bonds broken in this reaction has not been established, but in all probability, it is the phospho-ester bonds that are involved.

As with all degradative processes, the nature of the action of ultrasonic irradiation on deoxypentose nucleic acid is dependent on the energy and the time of irradiation. However, there is not complete agreement between the results of the studies that have been made concerning the type of bonds involved. Laland, Overend and Stacey[63] observed that the optical density at 259 mμ was not greatly affected by irradiation, a result later confirmed by Laland, Lee, Overend and Peacocke[64]. However, these studies were made on a deoxypentose nucleic acid sample which had already been subject to some denaturation, most probably by solution in water, as the initial values of $\epsilon(P)$ were given[64] as 7500 compared with the value of about 6600 accepted for native deoxypentose nucleic acid. The effect of the ultrasonic radiation on the native hydrogen bonded structure could thus not be established. From the electrometric titration studies of Lee and Peacocke[65], it was evident that some rupture of the hydrogen bonded structure occurred on irradiation, as the difference between the forward and backward titration curves of the sonicated material was less than for the native acid. However, denaturation was by no means complete and some internucleotide bonds were also broken. Doty, McGill and Rice[66], on the other hand, consider that ultrasonic radiation does not disturb the hydrogen bonded structure of the native acid but produces double-chain scission to yield true deoxypentose nucleic acid homologues. The evidence for the absence of any denaturation lay in the constancy of $\epsilon(P)$ before and after irradiation and in the constancy of the temperature at which heat denaturation occurred. Furthermore, electron-microscopic examinations of the sonicates[67] confirmed the absence of denaturation, but gave some indication of occasional single chain breaks. The disagreement between these results could be related to differences in the irradiation procedure since Doty, McGill and Rice took precautions not to dissolve the deoxypentose nucleate in water but only in salt solutions, and furthermore carried out irradiation in the absence of oxygen. In the earlier experiments[42, 63–65] these precautions were apparently not observed.

The effect of ionizing radiations, *viz.* x- and γ-rays, on solutions of deoxypentose nucleic acid has been known for some time to produce marked degradation, as generally indicated by a fall in viscosity, the loss of streaming birefringence and a decrease in molecular weight[68–75]. It has also been established that this degradation is an indirect effect due to the radicals, $OH\cdot$ and $HO_2^{\cdot}$ formed by the breakdown of water by the radiation in the presence of oxygen[69, 76–80]. The final over-all breakdown of deoxypentose nucleic acid, in terms of viscosity loss, for a given dosage of x-rays, however, has been found to be the same whether the irradiation is carried out in the presence or absence of oxygen[81] so long as the viscosity is determined sufficiently long after irradiation for the ‘ after effect ’ to be completed[73, 76, 77]. The effect of the dosage on the intrinsic viscosity has been investigated by Cox, Overend, Peacocke and Wilson[82, 83] and also by Butler, Pain, Robins and Rotblat[84]. The former authors showed that log $[\eta]$ is a linear function of log R, where R is the γ-ray dosage, at high values of R, whereas the latter authors observed a linear relationship between $[\eta]$ and dose for 140 kV x-rays and 15 MeV electrons. According to Cox, Overend, Peacocke and Wilson[82, 83], γ-irradiation produces a double-chain scission of the double helix at approximately opposite positions in the two polynucleotide chains as well as rupture of some of the specific hydrogen bonds. The disruption of the hydrogen bonds, as determined by electrometric titration and increase in the optical density at 259 mμ, is progressive, increasing with the dose.

These conclusions have been confirmed by Peacocke and Preston[33] who determined the molecular weight of the degradation products of deoxypentose nucleic acid produced by γ-radiation. According to the theory of the degradation of macromolecules[85, 86] the slope of the plot of log $[\eta]$ against log R should give a slope equal to $-\alpha$ in the equation $[\eta] = KM^{\alpha}$, where K is a constant and M the molecular weight. From the degradation studies, the slope was found[82, 83] to be $-1{\cdot}85$, whereas from molecular weight measurements on the degraded solutions α was found to have a value, 1·0, slightly more than half this value. This was interpreted by Peacocke and Preston[33] as evidence that it is a double and not a single chain that is being broken and thus confirms the mechanism of the γ-ray degradation as involving two independent and opposite breaks in the deoxypentose nucleic acid molecule. That the γ-radiation did not rupture all the hydrogen bonds before breaking covalent bonds was shown by the observation of a decrease in the molecular weight on heat denaturation of the γ-irradiated nucleate[33]. Apparently, on hydrogen bond rupture, the short polynucleotide chains of the degraded nucleate could diffuse apart. It may thus be concluded that in general the action of ionizing radiation is at first to bring about simultaneous partial denaturation and degradation of deoxypentose nucleic acid. More prolonged or intensive radiation produces much more complete breakdown such as deamination of the purines and pyrimidines, oxidation of the sugar and dehydroxylation.

Crystalline pancreatic deoxyribonuclease[87] after prolonged action on deoxyribonucleic acid yields the 5′-nucleotides, oligonucleotides and non-dialysable fragments. The use of deoxyribonuclease in the preparation of the nucleotides has already been discussed (Chapter 4) and only those aspects of the enzymic degradation which have a bearing on the structure of the native acid will be considered here.

The main features of the reaction of deoxyribonuclease on deoxyribonucleic acid which have been known for some time are the liberation of secondary phosphoric acid groups by breaking of the phospho-ester bond[88, 89], a decrease in the viscosity and the molecular weight[90, 91], and the formation of nucleotides and a ‘ core ’ resistant to attack by the enzyme[92, 93]. The early stages of the reaction between the enzyme and the nucleic acid are now more fully understood following careful studies of the molecular weight and viscosity changes during the reaction. At fairly high relative concentrations of deoxyribonuclease to nucleic acid (0·12 to 0·8μg of deoxyribonuclease to 27 to 28 mg of nucleic acid), Dekker and Schachman[61] found that whereas the viscosity decreased steadily with time, the optical density showed an induction period and did not change over the period of time in which the viscosity fell to about 7 per cent of its initial value. This was interpreted as indicating that rupture of the double helix occurred through cleavage of the phospho-ester bonds without affecting the hydrogen bonded structure.

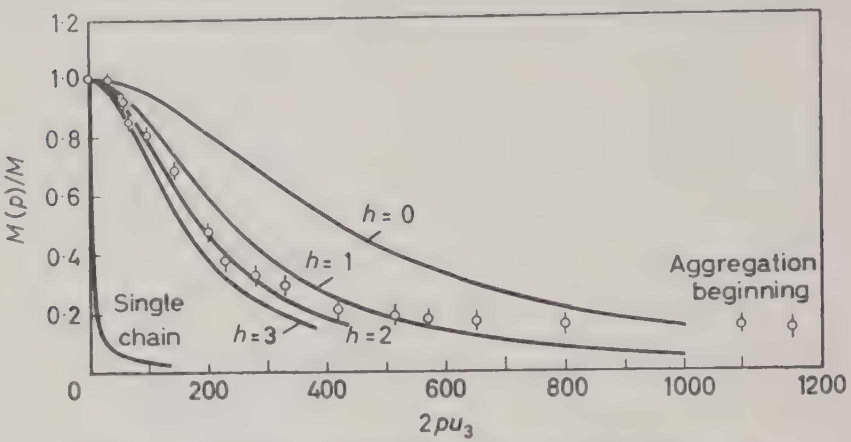


Figure 78. Decrease of molecular weight during enzyme action as a function of the number of bonds broken per parent *z*-average molecule

The points were determined by light scattering; the lines are calculated from double-chain degradation theory

(C. A. Thomas: *J. Amer. chem. Soc.* 1956, **78**, p. 1864)

At lower relative concentrations of deoxyribonuclease and nucleic acid (6×10^{-5}μg of deoxyribonuclease to 650μg of nucleic acid), Zamenhof, Griboff and Marullo[44] also observed an induction period for the fall in viscosity with time of enzyme action. That the fall in viscosity was due to a reduction of the molecular weight, and not to denaturation, thus confirming Dekker and Schachman's conclusions, was shown by Reichmann[94] by following the change in molecular weight with time by light scattering measurements. As in the measurements of viscosity and optical density, an induction period was observed, during which there was little change of molecular weight.

Owing to the liberation of secondary phosphoric acid groups, through rupture of the phospho-ester bonds, during the course of the deoxyribonuclease action, the pH of the solution changes as degradation proceeds. This makes the study of the kinetics difficult, since the change in pH of the solution will alter the shape of the deoxyribonucleic acid molecule, which will in turn produce changes in viscosity and light scattering behaviour not directly caused by the enzymic degradation. This difficulty was overcome by Thomas[95], who devised a method for maintaining the pH constant by automatically titrating the solution with alkali during the course of the enzyme reaction. By this means, not only was the pH of the reaction mixture kept constant but from the amount of alkali added at any given time the number of phospho-ester bonds broken could be calculated. In *Figure 78* is shown the fractional decrease of weight average molecular weight $[M(p)/M]$ as a function of the average number of bonds broken per parent (z-average) molecule. (The experimental values are represented by points in *Figure 78*. The quantity $2pu_3$ is defined as:

$$2pu_3 = \frac{\text{moles of NaOH required}}{\text{moles of nucleate present}} \times \frac{1}{0{\cdot}91}$$

The factor 1/0·91 arises from the assumption that the pK_a' value of the secondary phosphoric acid group is 6·0 and hence only 91 per cent of these groups is titrated by holding the pH constant at 7·0.) The curve for the viscosity decay is very similar. The induction period is followed by a rapid decline in molecular weight, reaching one-half its original value when 200 bonds per molecule have been broken. After 1200 bonds per molecule have been broken, a rapid aggregation of the fragments was invariably observed by Thomas[95]. This may be due to reformation of hydrogen bonds as occurs on neutralizing solutions of sodium deoxypentose nucleate taken to pH 2·6, and may also represent the core found by previous workers not to be attacked by deoxyribonuclease. The reason for the lack of enzymic degradation of the core would then be due to absence of the native structure.

If the deoxyribonucleic acid molecule is first degraded by deoxyribonuclease to yield fragments which still retain the specific hydrogen bonded structure, as is indicated by the marked induction period in the rise of optical density, phospho-ester bonds approximately opposite each other in both strands must be broken by the action of the enzyme (not necessarily simultaneously). If the hydrogen bonds are very strong, the enzyme must break an ester bond immediately opposite an already existing broken bond in order to cause a molecular scission. The probability of breaking both chains in such a way as to produce two fragments is:

$$s = p^2$$

where s is the probability of a double chain scission and p is the probability of a phospho-ester bond breakage. If the hydrogen bonds are weaker, then 1, 2 ... h contiguous hydrogen bonds may fail to hold the two fragments together. In this case the probability of a double chain scission is greater, being:

$$s = p[1 - (1 - p)^{2h+1}] \approx p^2(2h + 1)$$

In *Figure 78* is shown the calculated molecular weight decay for different values of h. The curve most closely following the experimental values is that for $h = 2$. It thus appears that the experimental results can be explained by assuming that the deoxyribonuclease breaks phospho-ester linkages at random and if these breaks appear on opposite chains not more than two hydrogen bonds apart, a scission of the molecule will result. If the breaks appear 3, 4, 5 or more hydrogen bonds apart, the molecule will hold together by the co-operative effect of the hydrogen bonds.

The general nature of the molecular weight decay also indicates that all linkages between possible nucleotide pairs are equally susceptible to enzymic cleavage. If this were not so and the enzyme could only break bonds between certain nucleotides, the probability of opposite, or nearly opposite, cuts in the molecule would be very much smaller. Furthermore, end selective double scission is not possible, for in this case about 3000 bonds per parent z-average molecule would have to be broken before the molecular weight would fall to 90 per cent of its original value in place of the observed value of about 100 bonds (*Figure 78*).

Complementary results to those described above have been obtained by Schumaker, Richards and Schachman[96] for the kinetics of the deoxyribonuclease action by the application of statistical theory to the viscosity and sedimentation coefficient data. The theory leads to the expression:

$$\log(1 - R) = n \log t + \text{constant}$$

where R is defined as the ratio of the observed intrinsic viscosity to the initial value at any time t, and n is the number of strands in the deoxyribonucleic acid molecule. Analysis of the viscosity results leads to a value of n of 1·47. Various possibilities have been examined which could explain this value being less than 2. These include the existence of single strand molecules in the preparation, the attack by the enzyme on both strands simultaneously and the existence of preformed breaks in the molecule on the basis of the discontinuous strand model of Dekker and Schachman[61]. If gaps were present in the molecule, however, the results show that these would occur only about once every 3000 nucleotides which is much less frequent than originally proposed by Dekker and Schachman.

For this reason, Schumaker, Richards and Schachman finally conclude that their results fit best the continuous two strand model and that, in agreement with Thomas (see above) the number of hydrogen bonds necessary to prevent a molecule from breaking when both strands have been broken is not more than six.

Deoxyribonucleic acids possessing transforming activity show a rapid decrease in activity as the enzymic degradation proceeds, thus clearly indicating the relationship between molecular weight and biological activity[44]. This dependence has been studied by Litt, Marmur, Ephrussi-Taylor and Doty[97] by determining the transforming activity of sonicated fragments. A critical molecular weight about 1×10^6 was found below which the transforming activity was lost. This critical value may refer to the minimum molecular weight for attachment to or incorporation into the genetic material of the cell.

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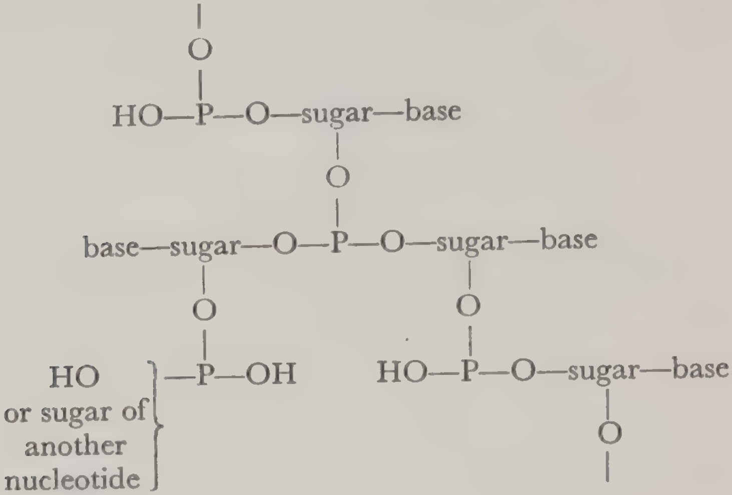
12

THE STRUCTURE OF NUCLEIC ACIDS: 5 THE STRUCTURE OF PENTOSE NUCLEIC ACIDS

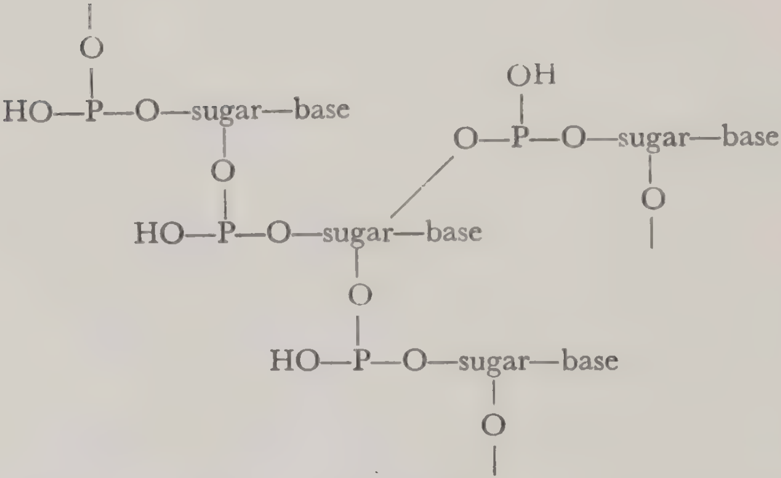
INTRODUCTION

FROM the evidence presented in Chapter 8, it was concluded that the predominating, if not the only, linkage in the pentose nucleic acids is a phosphodiester group joining the 3′ and 5′ positions of adjacent nucleotides. If this were the only form of the linkage, the pentose nucleic acids would be linear polynucleotides. However, suggestions have been made that a branched chain structure may occur in some, or all, pentose nucleic acids. There are two possible ways in which chain branching may occur. Firstly, through a phosphotriester linkage (*I*) and secondly, through the esterification of the $C'_{(2)}$ hydroxyl group of a pentose residue already esterified at $C'_{(3)}$ and $C'_{(5)}$ (*II*).

The evidence for the branched structure (*I*) involving a phosphotriester lay in the observation by Allen and Eiler[1] and Fletcher, Gulland and Jordan[2] that the electrometric titration curve of yeast ribonucleic acid showed the presence of approximately one group for every four atoms of phosphorus titrating in the range pH 5·0 to 7·5. This group was considered by Fletcher, Gulland and Jordan[2] to be a secondary phosphoric acid group which can arise in the polynucleotide by a chain branching of the type (*I*) since the nucleotide at the end of the side chain will necessarily possess a secondary, as well as a primary, unesterified phosphoric acid group. (The structure (*I*) involving a side chain is not the only one which conforms to the titration data, as a straight chain structure involving a triply esterified phosphorus atom and a $C'_{(3)}$—$C'_{(2)}$ linkage in place of a $C'_{(3)}$—$C'_{(5)}$ linkage was also suggested by Fletcher, Gulland and Jordan[2]. This structure, however, is subject to the same criticism as is given below of structure (*I*) and can also be rejected on the evidence presented in Chapter 8, which shows that the internucleotide linkage is predominantly $C'_{(3)}$—$C'_{(5)}$.) The shape of the electrometric titration curve of pentose nucleic acids has been confirmed by several workers[3–9] who have generally also interpreted their results by assuming the presence of secondary, as well as primary, phosphoric acid groups in the pentose nucleic acid. Likewise the binding of rosaniline, using the method of equilibrium dialysis[9, 10], has demonstrated the existence of two different dye binding sites on the pentose nucleic acid which were considered to be the primary and secondary phosphoric acid groups.


HO—P—O—sugar—base
base—sugar—O—P—O—sugar—base
HO or sugar of another nucleotide —P—OH
HO—P—O—sugar—base

(*I*)


HO—P—O—sugar—base
OH
O—P—O—sugar—base
HO—P—O—sugar—base
HO—P—O—sugar—base

(*II*)

In spite of this evidence supporting the existence of secondary, as well as primary, phosphoric acid groups in pentose nucleic acids, the conclusions have frequently been criticized as not representing the true nature of *native* pentose nucleic acid[11]. As has been pointed out in Chapter 4, the isolation of pentose nucleic acids from tissue without producing simultaneous degradation is difficult and it is almost certain that the earlier studies discussed above were all performed on degraded preparations. Degradation involving the hydrolysis of internucleotide phosphodiester bonds must inevitably liberate secondary phosphoric acid groups; the proportion of such groups found to be present will thus be dependent upon the nature of the extraction procedure and of any subsequent fractionation.

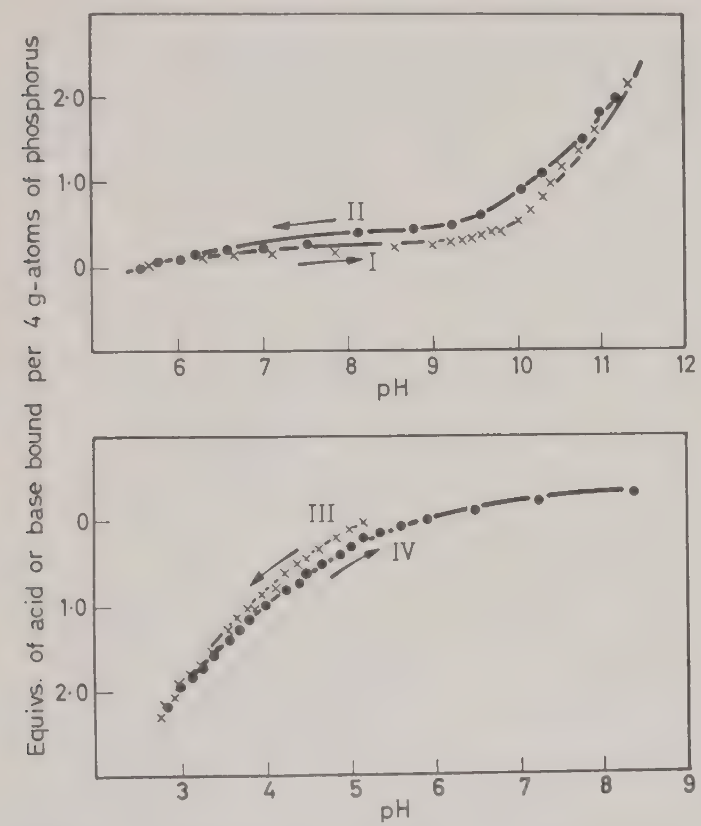


Figure 79. Titration curves for sodium ribonucleate from *Aerobacter aerogenes*

I and III, forward titration curves with alkali and acid respectively; II and IV, backward titration curves with acid and alkali respectively

(R. A. Cox, A. S. Jones, C. E. Marsh and A. R. Peacocke: *Biochim. biophys. Acta* 1956, **21**, p. 576)

This view has been confirmed by the titration of a preparation of yeast ribonucleic acid by Crestfield, Smith and Allen[12] which had been prepared by a relatively mild procedure (see Chapter 2, p. 31). Although the molecular weight of this specimen was not determined, it produced solutions possessing an intrinsic viscosity much higher than any values previously recorded. This sample of yeast ribonucleic acid showed very little buffer capacity in the pH range 6 to 8 and the authors concluded that it contained less than one secondary phosphoric acid group for every 16 atoms of phosphorus, which is a very much lower value than that observed by previous workers on less carefully prepared samples of pentose nucleic acids.

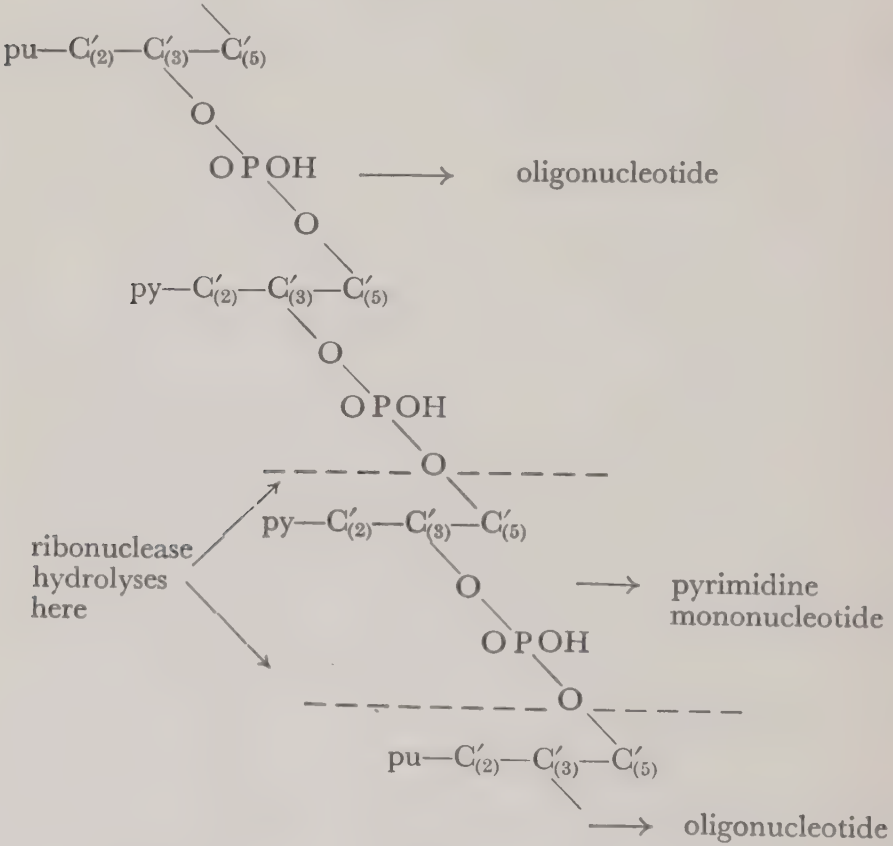
Further confirmation arises from the titration of the pentose nucleic acid from *Aerobacter aerogenes* by Cox, Jones, Marsh and Peacocke[13, 14] which had been extracted by a mild procedure (1 per cent sodium cholate in 0·14M sodium chloride at 60°C) and which possessed a molecular weight (determined by light scattering) of $1{\cdot}5 \times 10^5$, which is very much higher than that of the yeast ribonucleic acid studied by Fletcher, Gulland and Jordan[2] [$1{\cdot}5$–$2{\cdot}3 \times 10^4$ (determined from diffusion[15])]. The titration curves, which are shown in *Figure 79*, show the difference between the forward and backward titration curves which had previously only been observed with deoxypentose nucleic acids, and of more significance to the present discussion, an almost horizontal region in the range pH 5·0 to 7·5 in the forward and backward titration curves in the alkaline range (curves I and II, *Figure 79*). This clearly indicates that there is no appreciable amount of secondary phosphoric acid group present in this nucleic acid.

An essential feature of the chain branching postulated in structure (*I*) is the presence of a triply esterified phosphate group. If this group were to exist in pentose nucleic acids together with the diester group, the hydrolysis of the nucleic acid may well follow a complex course involving at least two different velocity constants. The course of the alkaline hydrolysis has been shown to be complex by both Bacher and Allen[16] and Cavalieri[17] and the latter author found it necessary to assume two velocity constants, one high and the other low, in order to explain satisfactorily the observed rate curve. However, this cannot be regarded as evidence in support of the presence of the phosphotriester group since the rates of hydrolysis of diester linkages between different pairs of nucleotides may not be equal, and furthermore the second stage may involve the hydrolysis of nucleotides to nucleosides and phosphate[16]. Of more significance is the observation by Brown, Magrath and Todd[18] that the triester, uridine-3′-dimethyl phosphate, is unstable at all pH values, the maximum stability occurring at pH 2–3 where it had a half-life of 4–5 hours. At pH 0 and 9 the half-life was approximately 15 minutes and at pH 7 about 75 minutes. In view of the fact that pentose nucleic acids are stable in solution at pH 7 and show no rapid degradation even at pH 10·6[19] Brown, Magrath and Todd[18] conclude that phosphotriester linkages cannot be present in the pentose nucleic acids.

In view therefore of the evidence presented above, and in particular of the absence of secondary phosphoric acid groups in carefully prepared specimens of pentose nucleic acid and of the instability of phosphotriesters in solution, it must be concluded that there are no satisfactory data which support structure (*I*) and it is to be assumed that phosphotriester linkages do not exist in isolated pentose nucleic acids. Whether such linkages exist in native nucleic acids in the living cell is likewise problematical in view of the instability of the phosphotriester linkage at pH 7, unless this is markedly influenced by the association of the nucleic acid with protein.

The second possible way in which chain branching may occur (structure *II*) was suggested by Brown and Todd[20] as conforming with the necessary condition for ease of hydrolysis, *viz.* the formation of a 2′—3′ cyclic triester (see Chapter 8, p. 145) and at the same time explaining the action of ribonuclease in preferentially liberating the pyrimidine nucleotides from pentose nucleic acid and leaving a non-dialysable ‘ limit polynucleotide ’ or ‘ core ’ which contained most of the purine nucleotide fraction of the original nucleic acid[9, 21–28]. Brown and Todd[20] thus developed the suggestion of Magasanik and Chargaff[28] that the pyrimidine nucleotides were mainly located in sidechains, of the type shown in (*II*) ; the polynucleotide ‘ backbone ’ would then be composed, predominantly, of purine nucleotides. However, this view of the structure of pentose nucleic acids requires considerable revision in view of the observation of Markham and Smith[29] and Volkin and Cohn[30] that the ‘ limit polynucleotide ’ or ‘ core ’ remaining after ribonuclease action, while not dialysing into water through cellophane, readily dialyses into salt solutions. These fractions have been found to be of much lower molecular weight than originally thought, being only three to five nucleotides in length. These observations, taken in conjunction with the now known basic mechanism of ribonuclease action (see below) to a large degree remove the evidence for the branched chain structure (*II*).

Pancreatic ribonuclease, as was first suggested by Schmidt and co-workers[27], and Loring, Carpenter and Roll[22], specifically breaks only the internucleotide bond between a 3′-pyrimidine nucleoside phosphoryl group and the 5′-hydroxyl group of an adjacent purine or pyrimidine residue. This specificity was successfully confirmed by Brown and Todd[31], who showed that ribonuclease readily hydrolysed the monoesters (methyl, ethyl or benzyl) of cytosine- and uridine-3′-phosphates but neither the corresponding 2′-phosphates nor the corresponding esters of either of the adenylic acid isomers, and by Brown, Dekker and Todd[32] and by Markham and Smith[33] who observed that ribonuclease readily attacks the cyclic 2′,3′-phosphates of uridine and cytidine but not the adenosine and guanosine analogues. These views are also confirmed by the action of ribonuclease on synthetic polynucleotides which will be discussed in Chapter 14. The production of pyrimidine mononucleotides in the ribonuclease digest may thus be explained by the presence in a *straight chain* polynucleotide of two or more adjacent pyrimidine nucleotides; the ribonuclease will specifically hydrolyse only those bonds cut by the broken line in (*III*) and thus only liberate a pyrimidine mononucleotide.


pu—C′(2)—C′(3)—C′(5)
O
OPOH
oligonucleotide
O
py—C′(2)—C′(3)—C′(5)
O
OPOH
O
py—C′(2)—C′(3)—C′(5)
ribonuclease hydrolyses here
O
pyrimidine mononucleotide
OPOH
O
pu—C′(2)—C′(3)—C′(5)
oligonucleotide

(*III*)

A dinucleotide will result from ribonuclease action at a site where a purine nucleotide is flanked on either side by a pyrimidine nucleotide, and larger oligonucleotides from positions where several purine nucleotides occur consecutively[29, 34].

It is therefore evident that it is not necessary to postulate a branched chain structure of the type (*II*) to explain the action of ribonuclease on pentose nucleic acids although it is true such a structure could equally well explain the observed results as a straight chain structure[29, 35].

Attempts to substantiate the presence of a branched chain structure have been made by the methylation of yeast ribonucleic acid followed by complete hydrolysis and separation and analysis of the methylated sugar derivatives. A straight chain structure, with a 3′—5′ phosphodiester linkage, would lead to the isolation of only 2′-methyl ribose, while branching of the type (*II*) would lead to the presence of ribose in the hydrolysate. Although ribose has been detected in the hydrolysate[36, 37] it is considered that this does not necessarily provide convincing proof of chain branching since it has been demonstrated[38] that phosphoryl migration occurs during the methylation procedure. This method is therefore unlikely to afford reliable evidence of structure when applied to polynucleotides, since under the experimental conditions, internucleotide bond fission will accompany methylation. It may thus be concluded that at present there is no direct evidence for or against the branched chain structure (*II*).

THE MOLECULAR CONFIGURATION OF THE PENTOSE NUCLEIC ACIDS

In marked contrast to the deoxypentose nucleic acids, knowledge of the molecular configuration of the pentose nucleic acids is almost rudimentary. Successful x-ray diffraction studies require oriented molecules, either in the form of crystals or fibres and although Rich and Watson[39] have obtained fibres of pentose nucleic acids by withdrawing a glass rod from a concentrated solution, as was done with deoxypentose nucleic acid (see Chapter 9, p. 162) the degree of molecular orientation was poor and sharply defined x-ray diffraction photographs were not obtained. This may be due to the fact that the pentose nucleic acid molecule is intrinsically shorter and consequently more difficult to align or because it is irregular in shape and does not pack well.

A typical x-ray diffraction diagram obtained by Rich and Watson[40] is shown in *Plate 6*. Identical photographs were obtained for the pentose nucleic acids from yeast, calf liver, calf thymus, tobacco mosaic virus and turnip yellow mosaic virus. Although this suggests that there is a unique structure of pentose nucleic acid which is independent of source[41], the possibility exists that were better defined photographs obtainable, discrete differences might be observed. A comparison with the x-ray diffraction photograph of deoxypentose nucleic acid may be made by comparing *Plate 6* with *Plate 1* (facing p. 162). Variation of the water content of the pentose nucleic acid fibres changes the x-ray diffraction pattern, which is reminiscent of the behaviour of deoxypentose nucleic acid although the pattern changes in a different way. The photograph shown in *Plate 6* was obtained on a dry fibre; if the relative humidity is raised above 90 per cent, the powder rings are sharpened and a new ring appears which is indicative of a long-range order. This change between ' wet ' and ' dry ' forms is reversible.

In spite of the poor definition of the x-ray diffraction patterns, a number of deductions may be made from them. There are strong reflections which correspond to a regular spacing of 3·3 to 4·0 Å along the fibre axis. These, in all probability, are associated with the stacking of purine and pyrimidine bases at right angles to the fibre axis, a conclusion supported by the negative birefringence of the fibres. The repeat distance along the fibre axis is 25 to 28 Å, a figure which is to be compared with 28 Å for the repeat distance in crystalline (A form) deoxypentose nucleic acid and 34 Å in the paracrystalline (B) form. There also appears to be a strong secondary repeat distance at 12 to 14 Å. There are no reflections on the meridian between about 13 Å and 4 Å, which has been interpreted by Rich[41] and Rich and Watson[39, 40] as indicating a helical structure. The equatorial reflections are not well resolved, so that it is difficult to determine the diameter of the helix with accuracy; however, this has been estimated[40, 41] as between 21 and 25 Å.

There thus appears to be a marked similarity between the dimensions of the pentose and deoxypentose nucleic acids. However, there is no definite evidence from the x-ray diffraction studies of the pentose nucleic acid whether this nucleic acid consists of a single helical polynucleotide strand or, like deoxypentose nucleic acids, contains two strands linked by specific hydrogen bonds. Evidence from other studies is likewise inconclusive and does not at present enable a definite decision to be made between these alternatives. Cox, Jones, Marsh and Peacocke[13] have observed (see *Figure 79*) an anomalous titration curve for the pentose nucleic acid isolated by a mild method from *Aerobacter aerogenes* very similar to that observed for deoxypentose nucleic acids (see *Figure 36*, p. 170). A similar titration curve was observed in the acid region

Plate 6. X-ray diffraction photograph of ribonucleic acid fibre (A. Rich and J. D. Watson: *Proc. nat. Acad. Sci., Wash.* 1954, **40**, p. 762)

(*To face page 284*)

for a preparation of yeast ribonucleic acid isolated by a mild procedure[14]. The difference between the forward and back-titration curves (*Figure 79*) is not as great as with deoxypentose nucleic acid, but is nevertheless outside the limits of experimental error and not due to contamination by deoxypentose nucleic acid. From the titration curve it appears[13] that approximately 25 to 30 per cent of the 6-amino groups and of the —NH—CO— groups of the nucleic acid are hydrogen bonded. In order to determine whether these hydrogen bonds link amino groups and the —NH—CO— groups, exact coincidence of the acid and alkali back-titration curves is necessary, as with deoxypentose nucleic acid. From the preliminary studies of Cox, Jones, Marsh and Peacocke[13] this point cannot be established precisely although the indications are that the two back-titration curves are not coincident. If this were substantiated, it would imply that a different type of hydrogen bonding occurs in pentose nucleic acid compared with that in deoxypentose nucleic acid. However, another explanation could be that, in view of the relatively small proportion of the amino and —NH—CO— groups taking part in the hydrogen bonds, the nucleic acid sample is a mixture of hydrogen bonded and non-hydrogen bonded molecules[42].

Further evidence for a double helical structure comes from a comparison of the x-ray diffraction diagram of pentose nucleic acid with that of synthetic polyadenylic acid (see Chapter 14). While the structure of polyadenylic acid is not known precisely, Watson[43] considers from the x-ray diffraction pattern that two polyadenylic acid chains are present in each unit cell. From a consideration of the various possible model structures and their predicted x-ray diagrams, it is furthermore considered very probable that these two chains are intertwined in a similar way to the two polydeoxynucleotide chains in the Watson and Crick structure for deoxypentose nucleic acid. Since there is a marked similarity between the x-ray diffraction patterns of polyadenylic acid and pentose nucleic acid, Watson concludes[43] that if polyadenylic acid possesses a two-strand hydrogen bonded helical structure, it is most likely that pentose nucleic acid does also.

An essential feature of the Watson and Crick structure for deoxypentose nucleic acid, is the hydrogen bonding between specific base pairs, guanine bonding to cytosine and adenine to thymine. The considerable analytical data on the deoxypentose nucleic acids isolated from various sources were used originally to support this specificity, although some doubt has recently been cast on the validity of this evidence by the variable values of the guanine to cytosine and adenine to thymine ratios observed in some fractions of deoxypentose nucleic acid (see *Table 1*, p. 53). However, in pentose nucleic acids, complementary ratios are rarely found (see *Table 7*, p. 91) although Rich and Watson[40, 41] have shown that in certain selected cases of pentose nucleic acids from mammalian tissues such ratios do occur. With the pentose nucleic acids of the plant viruses, however, no complementary ratios were found. Elson and Chargaff[44, 45] have regarded the failure to obtain complementary ratios in the pentose nucleic acids as due to the possibility that only part of the pentose nucleic acid of the cell was being analysed. They have therefore developed techniques[46–48] for isolating the total pentose nucleic acid of a cell. From these studies it is observed (see *Table 23*) that while the ratios of adenine to uracil and of guanine to cytosine do not equal unity, they nevertheless have approximately the same value. Important as these conclusions are, it must nevertheless be appreciated that the absence of complementary ratios in the pentose nucleic acids could be attributed to other causes. It could be attributed to a wide variation of base composition in the various nucleic acid fractions of which the total sample of nucleic acid is composed, each fraction possessing complementary ratios. Alternatively, it could be attributed to an irregularity in the hydrogen bonding; Watson[43, 49] has pointed out that if pentose nucleic acid possesses a structure like polyadenylic acid, the hydrogen bonding must be irregular. Lastly, the absence of complementary ratios could be explained either by a single strand helix or by a double helix with considerable

Table 23. Molar relationships for some pentose nucleic acids isolated from whole tissues and cells

Tissue	*Adenine*	*Guanine*	*Cytosine*	*Uracil*	$\frac{\text{AD}}{\text{UR}}$	$\frac{\text{GU}}{\text{CY}}$
Ox liver	17·10	27·30	33·90	21·70	0·79	0·80
Ox kidney	19·70	26·70	33·40	20·20	0·97	0·80
Rat kidney	19·40	29·50	30·70	20·40	0·95	0·96
Sea urchin	22·56	29·42	27·19	20·82	1·09	1·09
Yeast	25·40	24·60	22·63	27·37	0·93	1·09
Serratia marcescens	20·30	31·20	24·30	24·10	0·84	1·28
Escherichia coli	25·30	28·80	24·70	21·20	1·19	1·16
Mycobacterium phlei	20·85	30·75	27·10	21·25	0·98	1·13

(From D. Elson and E. Chargaff: *Biochim. biophys. Acta* 1955, **17**, p. 369) side chain formation, complementary hydrogen bonding occurring in the double helical ' backbone ' but not in the side chains.

For several viruses, which can be obtained in the crystalline form, it has been established by Franklin and co-workers[50–55] and by Ginoza[56] that the pentose nucleic acid in the virus is in the form of a single helix of radius 40 Å. This nucleic acid is embedded in the protein sub-units, which thus play an important part in determining the configuration of the pentose nucleic acid in the virus. Removal of the protein to give the free virus pentose nucleic acid will almost certainly produce changes in the dimension of the helix, but one would not have expected the single strand nature of the helix to have been altered. However, in view of the similarity of the x-ray diffraction diagram of the pentose nucleic acids of tobacco mosaic virus and of yeast observed by Rich and Watson[40, 41] and the possibility that the latter is a double stranded helix, the possibility that the tobacco mosaic virus nucleic acid changes from a single stranded helix to a double helix on extraction must not be altogether excluded. The ready formation of helical complexes, which may contain two or three like or unlike strands, by the synthetic polynucleotides (see Chapter 14) shows that such aggregation is not impossible.

Increases in the values of $\epsilon(P)$ with the action of various chemical reagents and enzymes on pentose nucleic acids[57, 58] cannot be regarded as a denaturation effect analogous to that observed with native deoxypentose nucleic acids, as the lowest values of $\epsilon(P)$ for native pentose nucleic acid are between 7700 and 7800 and correspond in magnitude to those of denatured deoxypentose nucleic acid. The similarity of these values is not surprising since the virus nucleic acids were isolated from the virus by a heat denaturation procedure. The pentose nucleic acid prepared from tobacco mosaic virus by a detergent procedure or by extraction with redistilled phenol shows a depression of about 15 to 22 per cent in the optical density on the addition of electrolyte[59]. However there would not appear to be the same relationship between native character and optical density in the virus nucleic acid as there is with deoxypentose nucleic acids, as a virus nucleic acid extracted with commercial phenol and which does not show the hypochromic shift on the addition of electrolyte, has been found to possess the same infectivity as the preparations which do show the change in optical density.

From the above discussion, it will be apparent that present views on the structure of pentose nucleic acids are somewhat confused. It would appear necessary for more studies of the titration and x-ray diagrams to be made on careful preparations, and very probably for such studies to be made on individual fractions of pentose nucleic acids before further progress can be made. The possibility also exists that the structure of pentose nucleic acids isolated from cell tissue may differ radically from those isolated from virus. The present evidence suggests that the former may consist of a double strand helix whereas the latter forms a single strand helix.

THE SIZE AND SHAPE OF THE PENTOSE NUCLEATE ION IN SOLUTION

Compared with the numerous studies of solutions of sodium deoxypentose nucleate, those on solutions of sodium pentose nucleate in solution have been very few. Furthermore the results which have been obtained on pentose nucleic acids of similar origin do not show the measure of agreement that has been attained with the deoxypentose nucleic acids. The reason for this difference lies in the difficulties met in extracting pentose nucleic acids from tissue and viruses since, in general, the methods involve the use of less mild conditions than are possible with the deoxypentose nucleic acids. In recent years, the study of virus pentose nucleic acids has become more fruitful in view of the work of Fraenkel-Conrat and co-workers and of Gierer and Schramm who have been able to isolate pentose nucleic acid from various viruses possessing an infectivity not far short of that of the original virus. Furthermore it has been established that this infectivity is to be attributed to all, or nearly all, the nucleic acid molecules and not just to a particular high molecular weight fraction[60, 61]. It may thus be concluded that such virus pentose nucleic acid preparations must represent the effectively unchanged native nucleic acid.

The molecular weight of pentose nucleic acids has been the subject of controversy since the time of the earliest measurements which were thought to support the now long discarded tetranucleotide hypothesis. The results of recent molecular weight determinations on preparations, which are thought to be largely undegraded, are summarized in *Table 24*. The values given all lie above 10^5 and there can be little doubt that the earlier recorded values of about 10^4 and less[15, 68–74] were all obtained on degraded samples. In 1942 Cohen and Stanley[64] showed how a pentose nucleic acid of high molecular weight can be isolated by heat denaturation of the virus. Their methods of isolation have since been refined and the now generally accepted value for the molecular weight of this nucleic acid is about 1·7 × 10^6. We thus see that at least some pentose nucleic acids possess a molecular weight not far short of that found for deoxypentose nucleic acids and if the tobacco mosaic virus pentose nucleic acid is proved to be a single strand helix, the molecular weights of the single chain polynucleotides in the two acids will be very close.

An investigation of the procedure for the preparation of tobacco mosaic pentose nucleic acid by Hopkins and Sinsheimer[67] has revealed that at least three factors influence the molecular weight of the resulting nucleic acid. These are: (*1*) the concentration of the virus when it undergoes heat denaturation, (*2*) the presence of phosphate ions in the solution, and (*3*) the rate of cooling after heating. High concentrations of virus and fast cooling yield high values of the molecular weight (greater than 2 × 10^6) suggestive of an aggregation, while a large number of different procedures involving the presence of phosphate buffer, yield the value of 1·70 ± 0·1 × 10^6, referred to above. This pentose nucleic acid molecule is highly asymmetric and possesses a length of about 1600 Å in water solution.

Table 24. Molecular weight of various samples of pentose nucleic acids

Source	*Molecular weight* × 10^{-5}	*Method*	*Reference*
Yeast	1·0–3·0	Sedimentation, intrinsic viscosity	61
Aerobacter aerogenes	1·5	Light scattering	13
Rat liver	2·64	Sedimentation	62
Beef liver	1–3	Sedimentation, intrinsic viscosity	61
Tobacco mosaic virus	2·0–2·9	Sedimentation, diffusion	63
Tobacco mosaic virus	1·5–1·8	Sedimentation, intrinsic viscosity	63
Tobacco mosaic virus	16	Sedimentation, intrinsic viscosity	64
Tobacco mosaic virus	10–20	Sedimentation, intrinsic viscosity	61
Tobacco mosaic virus	17	Light scattering	65, 66

The tobacco mosaic virus nucleic acid undergoes spontaneous degradation to give nucleates of lower molecular weight (0·59 to 0·70 × 10^5) which possess little or no infectivity[61]. The rupture of the molecule appears to follow a regular rather than a random pattern[61, 64] suggesting the presence, at regular intervals along the molecular chain, of weak internucleotide bonds or discontinuities of structure.

Solutions of the high molecular weight preparations of the pentose nucleic acid from tobacco mosaic virus and rat liver show relatively high viscosities[63, 64] which are dependent upon the velocity gradient[63] and it appears that the length of the ion varies considerably with changes in ionic strength. Hopkins and Sinsheimer[67] have shown from light scattering measurements that the pentose nucleate ion, originally 1600 Å in length, contracts to 1050 Å on the addition of electrolyte and Kawade and Kitamura[62] have concluded from viscosity measurements that at low ionic strengths the nucleate ion behaves in a similar manner to a flexible linear polyelectrolyte. At higher ionic strengths, however, the viscosity behaviour more closely resembles that of a fairly compact random coil. The solution behaviour of the pentose nucleate ion thus shows that it is more flexible than the deoxypentose nucleate ion, which lends support to the view that the ion possesses a single chain rather than a hydrogen bonded double strand structure.

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THE STRUCTURE OF NUCLEIC ACIDS: 6 THE NUCLEOTIDE SEQUENCE IN THE POLYNUCLEOTIDE CHAIN

INTRODUCTION

The realization that preparations of deoxypentose and pentose nucleic acids can be divided into fractions of different nucleotide composition has been of fundamental importance to studies of the sequence of nucleotides in the polynucleotide chain. Clearly, a determination of nucleotide sequence can be carried out only if it is certain that the particular nucleic acid fraction being investigated possesses only a single type of polynucleotide with all molecules having the same number of nucleotides in exactly the same sequence. However, as will be evident from the discussion in Chapter 3 (p. 38) the experimental methods have not yet been developed to the stage where fractionations of this accuracy can be carried out and until advances have been made in the fractionation procedure, studies of the nucleotide sequence must of necessity be of limited scope. Nevertheless, in much the same way that analysis has shown that nucleic acids isolated from different tissues have very different compositions and must therefore have different nucleotide sequences, it is relevant to examine the structure of the dinucleotides and oligonucleotides produced by enzymic and chemical hydrolysis, since by this means elementary information on the sequence of nucleotides may be obtained which will be of importance when it is possible to obtain homogeneous nucleic acid fractions. Another related problem is the presence and nature of a terminal phosphate group.

THE NUCLEOTIDE SEQUENCE IN DEOXYPENTOSE NUCLEIC ACIDS

The action of deoxyribonuclease on deoxypentose nucleic acids is to produce mononucleotides, nucleosides and further degradation products of mononucleotides, and oligonucleotides. The exact nature of the product depends upon the conditions and the time of digestion. Prolonged action brings about degradation of the mononucleotides before all the deoxypentose nucleic acid is converted into mononucleotides[1]. More usually the conditions are such that little mononucleotide degradation occurs and the digest contains 15 to 18 per cent of the original nucleic acid as oligonucleotides consisting mainly of di- and trinucleotides[2].

The first isolation of a dinucleotide was made by Sinsheimer and Koerner[3], who isolated, by ion-exchange chromatography, two dinucleotides, one containing only deoxycytidylic acid ([CC]) and the other containing deoxycytidylic and deoxyadenylic acids ([CA])* from the products of the action of pancreatic deoxyribonuclease on the deoxyribose nucleic acid of calf thymus. Their work was confirmed and extended by Smith and Markham[4, 5] who successfully separated the di- and trinucleotides by paper chromatography and electrophoresis. The separation of dinucleotides, trinucleotides and dinucleoside monophosphates from deoxyribonuclease digests of several different deoxypentose nucleic acids using ion-exchange chromatography, followed by their identification, has been successfully used by Sinsheimer[6, 7], de Garilhe and Laskowski[8], and Shapiro and Chargaff[9] to identify the various pairs of adjacent nucleotides occurring in the deoxypentose nucleic acids. The components of a di- or trinucleotide may be determined either by an analysis of the ultra-violet absorption spectrum[6], rate of migration on paper electrophoresis[5], or more directly by quantitative degradation to mononucleotides followed by chromatographic analysis of the mononucleotide mixture[6, 10, 11]. The molar ratios of the mononucleotides thus obtained should be integers if the fractionation procedure has been satisfactory. These methods only give the ratios of the component nucleotides in the di- or trinucleotide and do not indicate the molecular magnitude of the fraction. This may be determined by various methods: (*1*) removal of the terminal phosphate with phosphomonoesterase and measurement of the ratio of phosphorus released to total phosphorus[6]; (*2*) removal of the terminal phosphate and treatment of the residue with phosphodiesterase followed by fractionation of the resultant nucleosides and nucleotides[6]; (*3*) removal of terminal phosphate followed by analysis for purines, pyrimidines and phosphorus[5]. From the results of Sinsheimer[6] given in *Table 25* it is evident that all the possible nucleotide pairs (but not necessarily all the dinucleotide isomers) exist in the deoxypentose nucleic acids of calf thymus and wheat germ with the exception of [MM]. This means that in the polynucleotide chains of these nucleic acids fourteen out of the possible fifteen pairs of adjacent nucleotides occur. In assessing the significance of these results it must be remembered that the fractions given in *Table 25* only represent about one-sixth of the total digest; nevertheless the identification of nearly all the possible deoxynucleotide pairs is of considerable importance to the problem of nucleotide sequence. The most striking feature of the results given in *Table 25*, which was also observed by Smith and Markham[5], is the very large amount of 5-methyl deoxycytidylic acid associated with deoxyguanylic acid; the [MG] fraction of the thymus deoxyribose nucleic acid fraction accounts for more than 30 per cent of all the 5-methyl deoxycytidylic acid present.

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* Presentation of polydeoxynucleotide structures—The deoxyribonucleosides will be represented by the initials: A = deoxyadenosine; G = deoxyguanosine; C = deoxycytidine; T = thymidine; M = 5-methyl deoxycytidine. The position of the esterified phosphoric acid in mononucleotides, nucleoside-diphosphates, dinucleotides, *etc.* is indicated by p being placed at the right of the nucleoside symbol if it is linked to a 3′ hydroxyl of that nucleoside and at the left if it is on the 5′ hydroxyl group. When no distinction of sequential isomers is intended, the symbols of the nucleosides comprising the oligonucleotide are placed in square brackets. Thus pC is 5′-deoxycytidylic acid, Cp is 3′-deoxycytidylic acid, pCp is deoxycytidine-3′,5′-diphosphate; [CT] stands for a mixture of pTpC and pCpT or for either of the isomeric dinucleotides.

Table 25. The dinucleotides occurring in deoxypentose nucleic acids

Dinucleotide	*Calf thymus deoxyribose nucleic acid*		*Wheat germ deoxypentose nucleic acid*	
	Per cent of absorption at 260 mμ	Per cent of P	Per cent of absorption at 260 mμ	Per cent of P
[MM]	0	0	0	0
[MC]	0·02	0·03	0·11	0·17
[MT]	0	0	0·31	0·43
[MA]	0	0	0·43	0·41
[MG]	0·72	1·03	1·52	2·08
[CC]	0·82	1·11	0·48	0·63
[CT]	2·42	3·12	1·57	1·97
[CA]	3·50	3·32	2·43	2·23
[CG]	0·79	0·87	0·44	0·47
[TT]	1·28	1·38	1·11	1·16
[TA]	1·71	1·46	1·68	1·39
[TG]	2·41	2·77	1·97	2·20
[AA]	0·53	0·46	0·46	0·39
[AG]	1·44	1·17	1·15	0·90
[GG]	0·76	0·82	0·76	0·79
Total	16·40	17·54	14·42	15·22

(R. L. Sinsheimer: *J. biol. Chem.* 1954, **208**, p. 454)

In addition to the dinucleotides listed in *Table 25*, Smith and Markham[5] have identified the trinucleotides [ACT] and [AMT] in the digests of herring spermatozoon deoxypentose nucleic acid.

The dinucleotide fractions isolated by the methods described above may consist of mixtures of the isomeric dinucleotides, pXpY and pYpX, or of the pure isomers. The molar ratio of the isomeric dinucleotides in any dinucleotide fraction may be determined in the following way[7]: (*1*) isolation of the mixed isomeric dinucleotides by ion-exchange chromatography; (*2*) removal of the terminal phosphate groups of the mixed isomeric dinucleotides with prostatic phosphomonoesterase; (*3*) isolation of the resultant mixed isomeric dinucleoside monophosphate diesters by ion-exchange chromatography; (*4*) splitting of the mixed isomeric dinucleoside monophosphate diesters by purified venom phosphodiesterase; (*5*) recovery and identification of the mononucleotide (-5′-phosphate) fractions from the latter digest by ion-exchange chromatography. From the optical density of each nucleotide fraction and the known extinction coefficients of the nucleotides[6], the molar ratio (X/Y) of the two nucleotides (X and Y) can be calculated. This molar ratio will be equal to the molar ratio of the dinucleotide isomers pYpX and pXpY (pYpX/pXpY). This method is based upon the assumptions that the terminal phosphate group of the dinucleotides is attached to a 5′ position and not the 3′ position (*i.e.* that isomers of the type XpYp and YpXp are not formed as a result of deoxyribonuclease action) and that the venom phosphodiesterase always splits the 5′-3′ phosphodiester linkage at the 3′ position. These assumptions have been shown to be valid by model experiments[7].

Analysis of the dinucleotides obtained from calf thymus deoxypentose nucleic acid (*Table 25*) yields the molar proportions of the isomeric dinucleotides given in *Table 26*. It is evident with those dinucleotides containing a purine and a pyrimidine nucleotide that, with one exception, the isomers in which the phosphodiester group links the 5′ position on a purine nucleoside with the 3′ position on a pyrimidine nucleoside, the terminal phosphate being on the 5′ position of the pyrimidine nucleoside (*e.g.* pCpA) are in great excess over the isomer with the reverse sequence, and in two cases (pApC and pGpM) the latter type of isomer could not be detected. The exception is the dinucleotide [CG], where pGpC is more abundant in the digest than pCpG. The observed sequences are of significance not only to the structure of deoxypentose nucleic acid but also to the mechanism of deoxyribonuclease action and until the latter is more fully understood a complete interpretation of these data cannot be made.

An alternative approach to the problem of the sequence of nucleotides in the deoxypentose nucleic acids has been made by Shapiro and Chargaff[9, 12]. The presence of the 3′,5′-disphosphates of deoxycytidine and thymidine among the acid hydrolysis products of deoxypentose nucleic acids led Levene[13] to the conclusion that there could only be an alternating purine–pyrimidine nucleotide sequence in the nucleic acid. This interpretation was based on the much greater lability of purine nucleotides than of pyrimidine nucleotides to acid[14]. Brown and Todd[15] originally suggested that a certain amount of these diphosphates might be expected as a result of random fission of the internucleotide linkages; however, Dekker, Michelson and Todd[16] later considered that the amounts formed are too large to be produced in this way. Brown and Todd[17] have thus concluded that the presence of the diphosphates suggests a fairly even or random distribution of purine and pyrimidine deoxyribonucleotides in the nucleic acid. Shapiro and Chargaff[9] have made a careful examination of the rates of acid hydrolysis of a series of dinucleotides, isolated by ion-exchange chromatography from the deoxyribonuclease digests of deoxypentose nucleic acids, all having deoxycytidylic acid as one of the components, *viz.* [AC], [CC], and [TC]. [GC] was not studied since its behaviour was considered likely to be very similar to that of [AC] in view of the similarity in the rates of liberation of adenine and guanine from deoxypentose nucleic acid during the formation of apurinic acid. Of the three dinucleotides, [AC] showed the greatest lability to

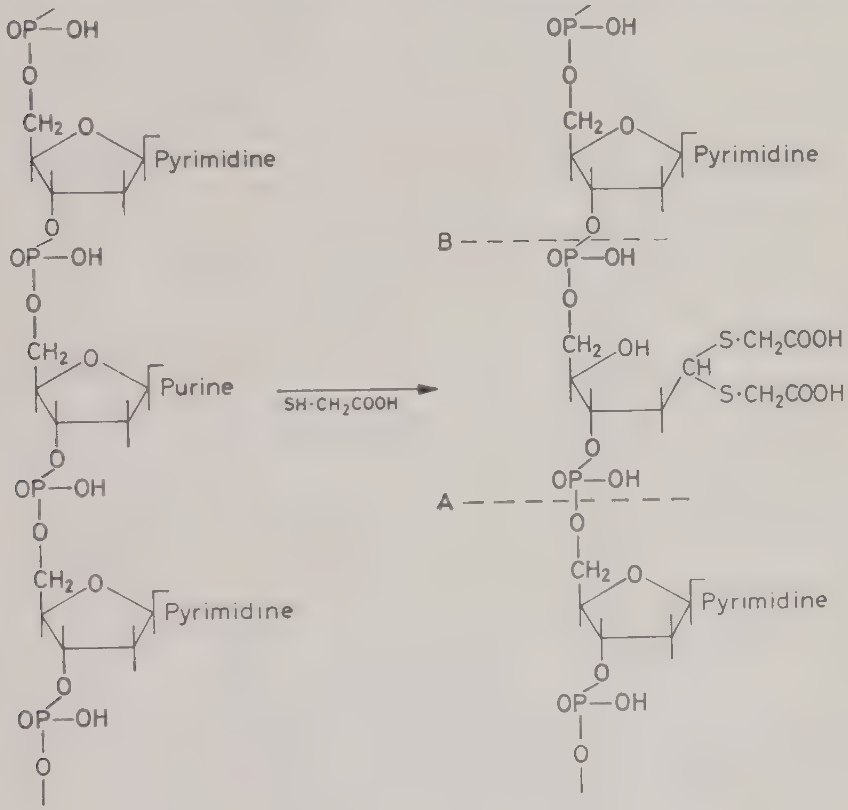
Table 26. Molar proportions of isomeric dinucleotides in calf thymus deoxypentose nucleic acid

Dinucleotide	Per cent of P	*Dinucleotide*	Per cent of P
pCpM	—	pMpC	—
pTpM	0	pMpT	0
pApM	0	pMpA	0
pGpM	0	pMpG	1·03
pTpC	0·78	pCpT	2·34
pApC	0	pCpA	3·22
pGpC	0·75	pCpG	0·12
pApT	0·10	pTpA	1·36
pGpT	0·16	pTpG	2·61
pGpA	0·97	pApG	0·20

(From R. L. Sinsheimer: *J. biol. Chem.* 1955, **215,** p. 581) acid. This dinucleotide is a mixture of the sequential isomers pCpA and pApC. Only the former isomer can hydrolyse to yield pCp and this it appears to do quantitatively. The latter isomer hydrolyses to give Cp and Ap. From a study of the hydrolysis of [CC], *i.e.* pCpC, it was found that the diphosphate pCp was formed preferentially, thus indicating that, of the two cytidine moieties, the one that is monophosphorylated is more labile to acid than the diphosphorylated nucleoside. In a similar way, from a study of the hydrolysis of the dinucleotide [TC], which consists of a mixture of the sequential isomers pCpT and pTpC, it was concluded that, of the glycosidic pyrimidine nucleoside bonds, that involving cytosine is more labile than that involving thymine[7].

The conclusions reached on the hydrolysis behaviour of dinucleotides have been used by Shapiro and Chargaff[12, 18] to interpret the acid hydrolysis of deoxypentose nucleic acid. Hydrolysis was carried out in 0·1M sulphuric acid at 100°C and the hydrolysate analysed at time intervals (30, 60 and 120 minutes) so judged as to achieve a separation in time of the two contributing hydrolytic reactions: (*1*) the comparatively rapid production of pyrimidine nucleoside diphosphates originating from pyrimidine nucleotide flanked on either side by purine nucleotides; and (*2*) the slower formation of diphosphates originating from pyrimidine nucleotides adjacent to one or two other pyrimidine nucleotides. From a study of several different nucleic acids, Shapiro and Chargaff concluded that rather than a regular distribution of the purine and pyrimidine deoxynucleotides in the nucleic acid, the evidence favoured the existence of polypyrimidine deoxynucleotide and polypurine deoxynucleotide regions in the polynucleotide chain. Furthermore, the evidence indicated that either the average length of the polydeoxycytidylic acid sequences was greater than the polythymidylic acid sequences or they occurred more frequently. These results are in conformity with those of Cohn and Volkin[19] who, by means of ion-exchange chromatography, isolated from the acid hydrolysate of deoxypentose nucleic acid all possible varieties of mono-, di- and trinucleotide sequences of deoxycytidylic and thymidylic acids.

The presence of polypyrimidine deoxynucleotide sequences in deoxypentose nucleic acids had been suggested, prior to the work of Shapiro and Chargaff, and Cohn and Volkin, by Tamm, Shapiro, Lipshitz and Chargaff[20] from a study of alkaline degradation products of apurinic acid. Apurinic acids, as their name suggests, are produced by the removal of all purine residues from the polynucleotide through fission of the acid labile purine N-glycosidic linkage and thus have deoxypentose phosphate residues in place of the purine deoxynucleotide residues originally present in the nucleic acid. Although the original products of mild acid hydrolysis were highly degraded, Tamm, Hodes and Chargaff[21] have been able to obtain substances having molecular weights of about 15,000. If instead of removing the purines from deoxypentose nucleic acid, as in the formation of apurinic acid, they are replaced



(I)

by carboxymethylmercaptal groups, the deoxypentose nucleic acid derivative thus formed would have the deoxypentose moieties, which were originally bound to the purines, in the open-chain form (see *I*). An hydroxyl group would thus be adjacent to the 3′- and 5′-phospho-ester groups, and according to the mechanism for the alkaline hydrolysis of pentose nucleic acids (see Chapter 8), such a structure should be labile to alkali and hence by alkaline degradation, fragments containing the pyrimidine nucleotide portions intact should be obtained.

Jones and Letham[22, 23] have treated the deoxyribose nucleic acid of calf thymus with mercaptoacetic acid in the presence of anhydrous zinc chloride and sodium sulphate, and found that the purines are replaced quantitatively by carboxymethylthio-groups to yield the aldehydoapurinic acid di(carboxymethyl)thioacetal (*I*). Alkaline hydrolysis of this substance with dilute alkali yields dialysable fragments, which have been separated by paper chromatography and electrophoresis by Jones and Letham[22], and Jones, Letham and Stacey[24]. Many of these fragments were identified as di- and tripyrimidine deoxynucleotide derivatives. These results thus conform with those of the acid hydrolysis of deoxypentose nucleic acid in indicating the presence, in the polynucleotide chain, of polypyrimidine deoxynucleotide sequences. A comparison of the components of the alkaline hydrolysate of the aldehydoapurinic acid di(carboxymethyl)dithioacetal derived from the deoxypentose nucleic acids of calf thymus and *Mycobacterium phlei* shows marked differences in the polypyrimidine deoxynucleotide parts of the two nucleic acids[25]. An alternative method of removing the purines and at the same time producing the liberated reducing sugars in the acyclic form has been described by Kent, Lucy and Ward[26], who obtained a diethyl dithioacetal from herring-roe deoxypentose nucleic acid by the use of ethanethiol and hydrochloric acid. Greater degradation of the nucleic acid was produced by this method compared with the reaction with mercaptoacetic acid. Nevertheless, polypyrimidine deoxynucleotides were detected in the hydrolysis products.

To summarize the above conclusions on nucleotide sequences in deoxypentose nucleic acids, obtained by three different methods, it seems fairly certain that each deoxynucleotide has somewhere along the polynucleotide chain, all other deoxynucleotides adjacent to it. Also there is direct evidence that pyrimidine deoxynucleotides are present as polypyrimidine deoxynucleotides or ‘ bunches ’ of at least three nucleotides in length, and because of both the complementary nature of pyrimidines and purines in the hydrogen bonded double helix and from the composition of the nucleic acid, the purines must occur in similar polypurine deoxynucleotides. These results have all been obtained on unfractionated deoxypentose nucleic acids and it is doubtful if such studies on individual fractions would lead to information of any greater value. The only study of fractionated material is that of Hodes and Chargaff[27, 28] who isolated apurinic acids from deoxypentose nucleic acid fractions obtained by the fractional dissociation of nucleohistone (see Chapter 3). The fractions were also hydrolysed with pancreatic deoxyribonuclease and the non-dialysable fractions analysed. The only consistent change with all three fractions was a relative depletion of all residues in cytosine. This work was of a very preliminary nature and further examination of more highly fractionated material is desirable.

An interesting recent development, which may well be of significance in sequence studies, is the observation by Razzell and Khorana[29] (see also Heppel and Rabinowitz[30]) that purified snake venom diesterase degrades pentathymidine tetraphosphate (TpTpTpTpT, prepared by the chemical polymerization of thymidine-5′-phosphate, followed by removal of the terminal phosphate group with phosphomonoesterase) in a stepwise manner starting from the end bearing the unesterified 3′-hydroxyl group. The diesterase from calf spleen, on the other hand, degrades this molecule also in a stepwise fashion, but from the other end, *i.e.* that bearing the free 5′-hydroxyl group.

The observation that the purine and pyrimidine deoxypentose nucleotides occur in the nucleic acids in polypurine and polypyrimidine sequences lays emphasis on the frequency with which such polymer groups will occur in a random polymerization. Calculation of pyrimidine sequences, on the basis of a random distribution, has been made by Peacocke (in Jones, Stacey and Watson[25]) in the following manner. Consider a large number of linear arrays each containing $N/2$ purines and $N/2$ pyrimidines, in which each site has an equal chance of being occupied by a purine or a pyrimidine. This latter condition corresponds to the requirement that in deoxypentose nucleic acids, the sum of the numbers of adenine and guanine residues must equal the sum of the numbers of cytosine and thymine residues.

The number of sequences of n pyrimidines will be $N/2^{n+2}$, *i.e.* the number of sequences of one, two, and three pyrimidines will be $N/2^3$, $N/2^4$, and $N/2^5$ respectively. The total number of pyrimidines occurring in all unbroken sequences of length n will be $Nn/2^{n+2}$. If $N \gg n$ and there is a very large number of arrays of N units, the total number of pyrimidines in a given array of N will, as first assumed, be, on average:

$$N\sum_{0}^{\infty} n/2^{n+2} = \tfrac{1}{2}N$$

Hence the fraction of pyrimidines occurring singly ($n = 1$) will be $N/2^3 \div N/2 = \frac{1}{4}$, and the fraction occurring in pairs ($n = 2$) will be $2N/2^4 \div N/2 = \frac{1}{4}$. Therefore the fraction of pyrimidines occurring in sequences greater than two (*i.e.* $n > 2$) $= \frac{1}{2}$.

Let the fraction of pyrimidines which are cytosine and thymine be c and t respectively. Then in deoxypentose nucleic acid $c + t = 1$. For single units of pyrimidines the fraction of pyrimidine occurring as cytosine will be $c/4$ and those occurring as thymine will be $t/4$.

For units of two pyrimidines, $c^2/4$ will occur in the sequence CC, $2ct/4$ in CT, and $t^2/4$ in TT. For units containing n nucleotides the proportions of the various arrangements can be obtained by the expansion of $(c + t)^n = 1$.

The existence of polymer units of one kind of nucleotide is thus to be expected in a polynucleotide; their existence therefore is not in itself of such great importance as their existence in other than the theoretical amounts. That the amounts do differ from the theoretical and depend on the source of deoxypentose nucleic acid is shown in *Table 27*.

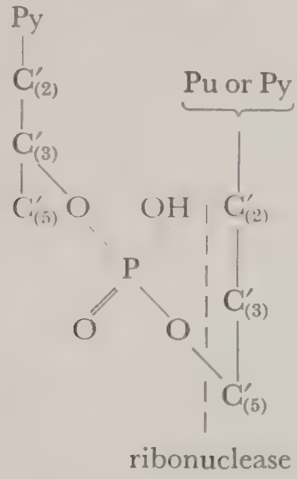
Table 27. Components of the alkaline hydrolysate of the aldehydo-apurinic acid di(carboxymethyl)dithioacetal of *M. phlei* and of calf thymus

Pyrimidine sequence	*M. phlei*		*Calf thymus*	
	Obs.	Theor.	Obs.	Theor.
T	2·0	7·8	3·0	13·8
C	4·0	17·3	2·0	11·3
TC	3·0	10·6	4·5	12·4
TT	0	2·5	2·0	7·6
CC	1·5	11·9	1·5	5·1

(A. S. Jones, M. Stacey and B. E. Watson: *J. chem. Soc.* 1957, p. 2455)

THE NUCLEOTIDE SEQUENCE IN PENTOSE NUCLEIC ACIDS

The study of the nucleotide sequence in pentose nucleic acids has followed very closely the methods described above for deoxypentose nucleic acids, except that no pentose analogue of apurinic acid exists. Pancreatic ribonuclease (ribonuclease I) has been shown by Schmidt and co-workers[31–33] to be highly specific and to hydrolyse only those internucleotide bonds between a phosphoric acid group on the 3′ position of a pyrimidine nucleoside and the 5′-hydroxy group of the adjacent purine or pyrimidine nucleotide, *i.e.* the rupture occurs specifically in the position shown in (*II*). Although Markham and Smith[34, 35], and Volkin and Cohn[36] have since shown that the 2′,3′-cyclic phosphates are formed as intermediates during ribonuclease action, this fact does not alter the specificity of the hydrolysis shown in (*II*). Prolonged action of ribonuclease on pentose nucleic acids liberates about 60 per cent of the pyrimidines as the 2′- or 3′-mononucleotides, the remainder of the pyrimidine nucleotides remaining attached to purine nucleotides or polynucleotides through the 5′ position (see structure (*III*), Chapter 12, p. 282). In view of the specific action of ribonuclease, it is evident, as was first pointed out by Schmidt and co-workers[32], that the liberation of pyrimidine mononucleotides in the ribonuclease digest must indicate that a high proportion of these nucleotides exist in the nucleic acid in polypyrimidine nucleotide

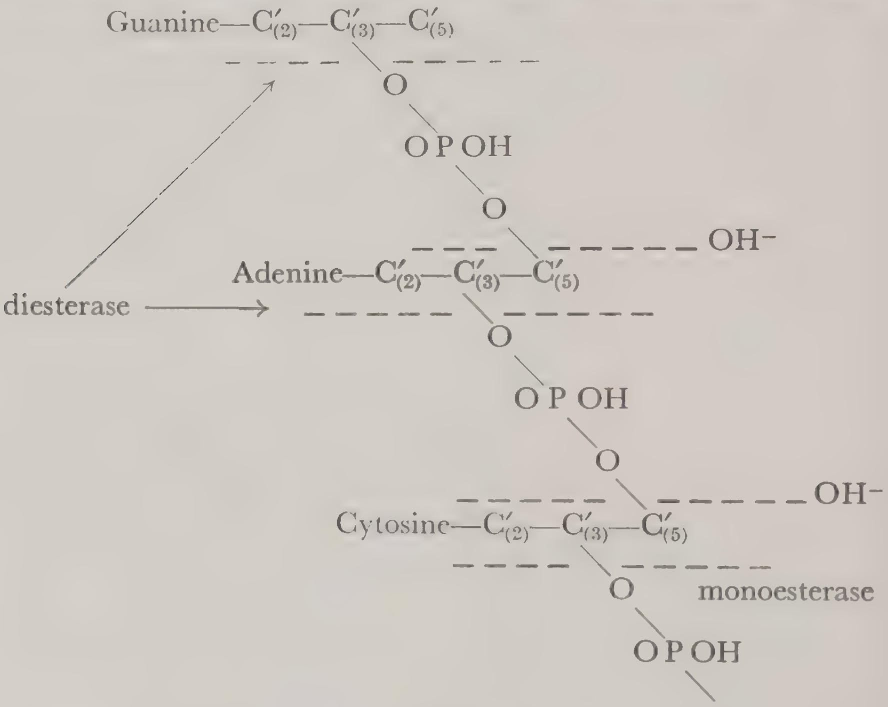


(*II*)

‘ blocks ’ or ‘ bunches.’ For example, if all the pyrimidine nucleotides existed in the nucleic acid as dipyrimidine nucleotides, 50 per cent of the pyrimidine nucleotides would be liberated on ribonuclease action. The fact that about 60 per cent are liberated must indicate that some ‘ blocks ’ must be more than two nucleotides in length. The fact that the pyrimidine nucleotides occur in the nucleic acids in this way, when taken in conjunction with the known composition, lead also to the conclusion that the purine nucleotides must occur in a similar ‘ bunched ’ form.

The oligonucleotides which are produced as a result of ribonuclease action and which will contain all the purine nucleotides with a pyrimidine end group, have been successfully fractionated by Markham and Smith[35] (see Chapter 4, p. 86) using paper chromatography and electrophoresis, and by Volkin and Cohn[36] by ion-exchange chromatography. A typical separation of the oligonucleotides of the pentose nucleic acid from calf liver by ion-exchange chromatography (Dowex 1 column, 2 per cent cross linking) is shown in *Figure 80*. The separated di- and trinucleotides, and in certain cases, oligonucleotides of higher orders may be identified by monoesterase, diesterase and alkaline hydrolysis in a somewhat similar manner to that already described for the dideoxynucleotides. However, since the oligopentose nucleotides are hydrolysed by alkali, whereas the oligodeoxynucleotides are not, the procedure is somewhat different and will be described with reference to a specific example[36].

The action of monoesterase, diesterase and alkali on the trinucleotide GpApCp* is shown in (*III*). Hydrolysis with alkali will yield guanylic and adenylic acids as a mixture of the 2′ and 3′ isomers, and cytidylic acid as the 3′ isomer only. Removal of the terminal phosphoric acid group with monoesterase yields a


Guanine—C′(2)—C′(3)—C′(5)
O
O P OH
O
OH⁻
Adenine—C′(2)—C′(3)—C′(5)
diesterase
O
O P OH
O
OH⁻
Cytosine—C′(2)—C′(3)—C′(5)
O
monoesterase
O P OH

(*III*)

---

* For the presentation of the polynucleotide structures, the same nomenclature will be used as for the polydeoxynucleotides. Thus the nucleosides will be represented by the initials: A = adenosine; G = guanosine; C = cytidine; U = uridine. The position of the esterified phosphate group is indicated by p being placed at the right of the nucleoside symbol if linked to a 3′ hydroxyl of that nucleoside, and to the left if it is on the 5′ hydroxyl group. When no distinction of sequential isomers is intended, the symbols of the nucleosides comprising the oligonucleotide are placed in square brackets.

product which on hydrolysis with alkali gives cytidine, and adenylic and guanylic acids as the 2′ and 3′ isomers. Hydrolysis of this same product with diesterase yields guanosine together with the -5′-phosphates of adenosine and cytidine. These degradations make it possible to define the structure of all the di- and trinucleotides produced by ribonuclease action and also the structure of certain higher polynucleotides.

An alternative procedure for identifying di- and trinucleotides has been developed by Whitfield and Markham[37, 38], and a similar procedure was independently suggested by Brown, Fried and Todd[39], for the determination of nucleotide sequence. The steps in the method as used for the determination of the structure of a di- or trinucleotide are given in (*IV*). The terminal phosphate group is first removed by a monoesterase; this leads to the presence of free hydroxyl groups in the 2′ and 3′ position of one of the pentose groups in the dinucleoside monophosphate produced. Oxidation with sodium periodate gives the dialdehyde derivative

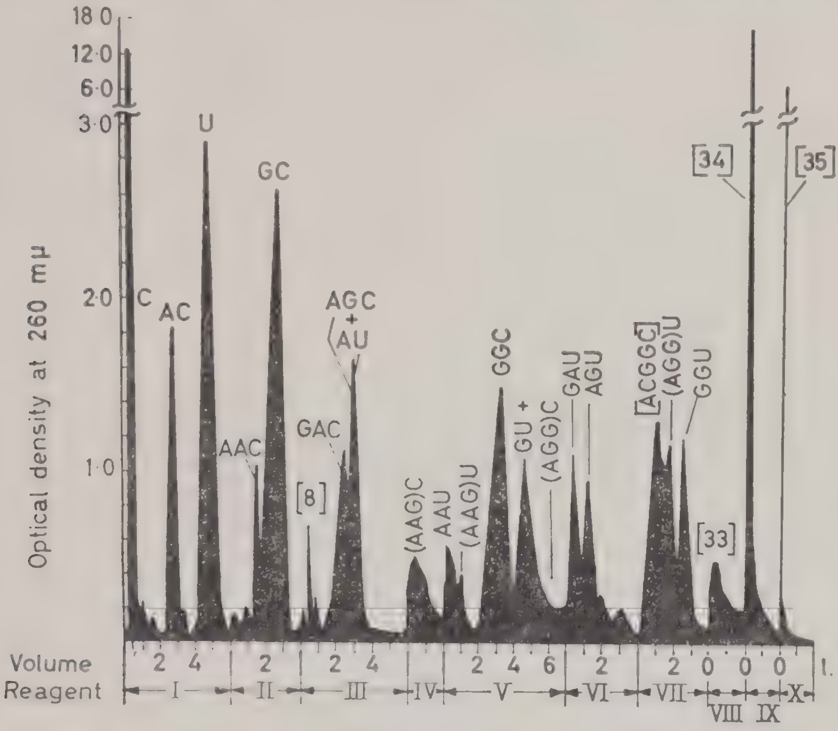
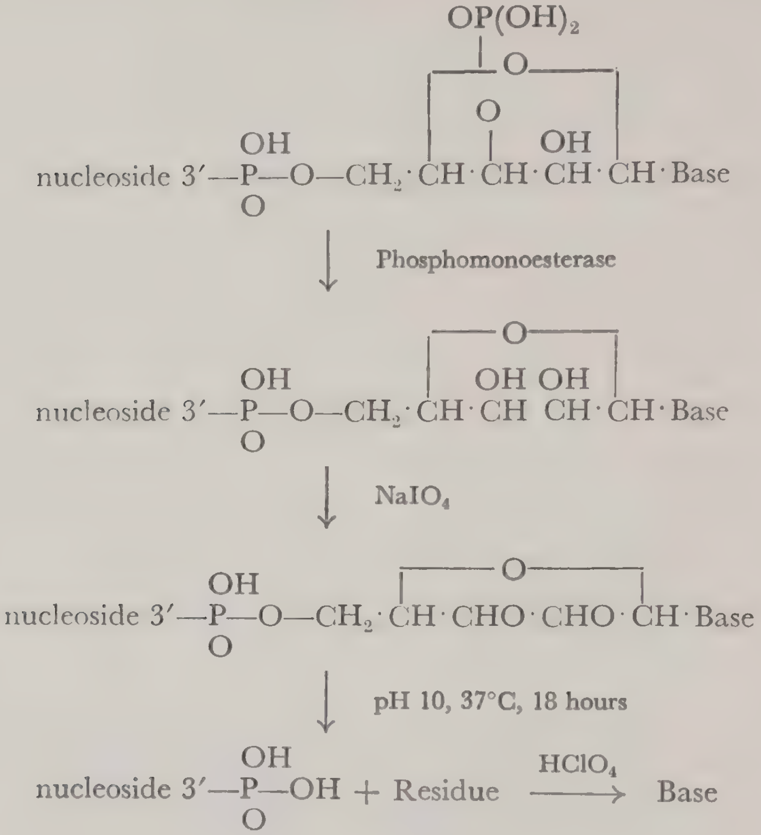


Figure 80. Ion-exchange separation of the products of ribonuclease digestion of the ribonucleic acid of calf liver
(E. Volkin and W. E. Cohn: *J. biol. Chem.* 1953, **205**, p. 774)

which on hydrolysis at pH 10 at 37°C for 18 hours, yields a nucleotide and a compound which on hydrolysis with perchloric acid gives a free pyrimidine or purine base. Identification of the nucleotide and the base gives the structure of the dinucleotide.


OP(OH)$_2$
O
O
OH
OH
nucleoside 3′—P—O—CH$_2$·CH·CH·CH·CH·Base
O
Phosphomonoesterase
O
OH
OH OH
nucleoside 3′—P—O—CH$_2$·CH·CH CH·CH·Base
O
NaIO$_4$
O
OH
nucleoside 3′—P—O—CH$_2$·CH·CHO·CHO·CH·Base
O
pH 10, 37°C, 18 hours
OH
nucleoside 3′—P—OH + Residue
O
HClO$_4$
Base

(*IV*)

This method could clearly be used on any homogeneous preparation of a polynucleotide to bring about a stepwise degradation and identification of the nucleotides through the bases liberated. The method, however, has not been used extensively for determining the structure of di- and trinucleotides, and for the majority of structural determinations the method based on enzymic and alkaline hydrolysis has been used.

Since there are four nucleotides in pentose nucleic acids, there are sixteen possible nucleotide sequences in the dinucleotides. Theoretically these sixteen sequences permit the existence of thirty-two dinucleotides, two for each sequence. Thus for the dinucleotide formed between adenylic and cytidylic acids (3′ and 5′ isomers) we have the following isomers: pCpA, CpAp, pApC, ApCp. Of these, only the last will be produced by the action of ribonuclease on pentose nucleic acids since the terminal phosphate must be on the 3′ (or 2′) position of a pyrimidine nucleoside. Markham and Smith[35] and Volkin and Cohn[36] have therefore found only the dinucleotides: ApCp, GpCp, ApUp, and GpUp in the ribonuclease digests. However, four other dinucleotide sequences, *viz.* ApGp, GpAp, GpGp, and ApAp were identified in the trinucleotides: ApGpUp, ApGpCp, GpApUp, GpApCp, GpGpUp, GpGpCp, ApApUp, ApApCp which were also isolated from the ribonuclease hydrolysate. The remaining eight possible nucleotide sequences have been obtained by Merrifield and Woolley[40], and Cohn and Markham[41] from the acid hydrolysates of yeast ribonucleic acid. The nucleic acid was treated for 2·5 minutes with 6M hydrochloric acid at 20°C, neutralized and the polynucleotide fractions separated either by ion-exchange[40] or paper chromatography[41]. The acid hydrolysis apparently ruptures internucleotide bonds at random irrespective of whether they involve purine or pyrimidine nucleotides. Breakage occurs at the same position as with ribonuclease, *i.e.* at the bond between a 3′-phosphoric acid group and the 5′-hydroxyl group of the adjacent nucleotide. Thus Merrifield and Wooley isolated the dinucleotides ApCp, CpAp, GpCp, CpCp, and [UC][40], and Cohn and Markham[41] identified the remaining sequences UpAp, CpGp, UpGp, and UpUp.

The study of the sequence of nucleotides in virus pentose nucleic acids is of particular significance in view of the infectivity of the nucleic acid itself and the homogeneity of the virus nucleic acid compared with that of nucleic acids extracted from tissue. Reddi[42] has recently studied the structure of the oligonucleotides produced by pancreatic ribonuclease and micrococcal phosphodiesterase digestion of various strains of tobacco mosaic virus. As with other pentose nucleic acids, the digestion of tobacco mosaic virus nucleic acid with ribonuclease liberates pyrimidine mononucleotides[43]. The pyrimidine nucleotides must therefore exist as polypyrimidine nucleotide ‘ bunches ’ in the polynucleotide chain. Only three dinucleotides, ApCp, ApUp and GpUp and the trinucleotide ApGpCp were isolated from the digests, although other trinucleotides were most probably present but not isolated. With micrococcal phosphodiesterase digests, the dinucleotides ApCp, ApUp, ApGp, ApAp, UpGp, UpCp, and UpUp were found, although the structure of these sequences could not be determined with certainty since the hydrolytic specificity of this enzyme is not known.

Of particular interest are the relative amounts of the nucleotide sequences in the nucleic acids from different strains of virus. The three strains TMV, HR, and M all possess the same base composition, but the amounts of cytidylic acid and uridylic acid liberated by prolonged ribonuclease digestion are different in the three strains (*Table 28*) indicating that the ‘ bunched ’ arrangement of the pyrimidine nucleotides in the three strains is different. Furthermore the amounts of the three dinucleotides and the trinucleotide isolated from the digest of the three virus nucleic acids are significantly different (*Table 29*). This confirms that the arrangement of the nucleotides in the three different strains of virus nucleic acid is different and represents the first direct evidence supporting the view that the differences in the biological activity

Table 28. The amounts of cytidylic acid and uridylic acid in the pancreatic ribonuclease digests of the pentose nucleic acids of different strains of tobacco mosaic virus

Strain	*Mononucleotide as percentage of total nucleotide in nucleic acid*	
	Cytidylic acid	*Uridylic acid*
TMV	45·7	50·7
HR	42·0	48·1
M	61·2	70·8

(From K. K. Reddi : *Biochim. biophys. Acta* 1957, **25**, p. 531)

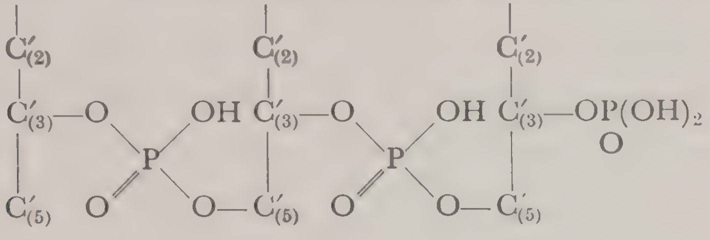
Table 29. The amounts of di- and trinucleotides in the pancreatic ribonuclease digests of the pentose nucleic acids of different strains of tobacco mosaic virus
(in μg/mg of nucleic acid)

Strain	ApCp	ApUp	GpUp	ApGpCp
TMV	39·4	44·8	47·6	85·3
HR	35·6	40·8	55·1	72·9
M	44·3	46·8	48·9	72·6

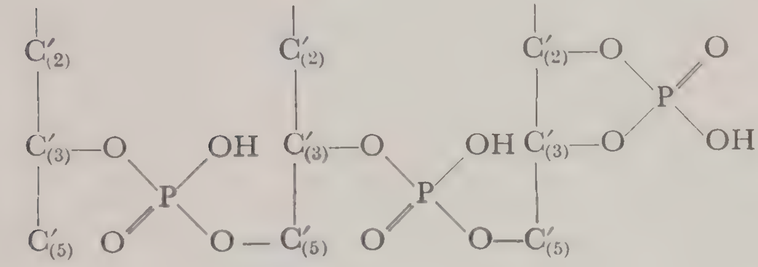
(K. K. Reddi : *Proc. nat. Acad. Sci., Wash.* 1959, **45**, p. 298) of viruses lie in the arrangement of the nucleotides in the polynucleotide chain.

THE TERMINAL PHOSPHATE GROUP

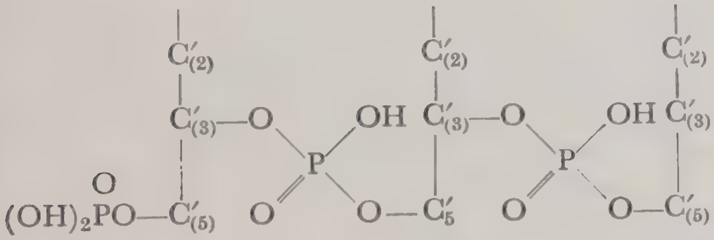
The terminal phosphate group, if present, may be located on a 3′ (or 2′) position (*Va*) or on a 5′ position (*Vc*) at the other end of the polynucleotide chain. If it is located at the 3′ (or 2′) position the possibility also arises of it existing in the 2′,3′ cyclic form (*Vb*). If these formulae (*V*) represent the true structures of homogeneous



(*Va*)



(*Vb*)



(*Vc*)

nucleic acids, structures (*Va*) and (*Vb*) could be considered as being formed from nucleoside-3′-phosphates and structure (*Vc*) from nucleoside-5′-phosphates. The presence of the structures in pentose nucleic acids may be detected by analysis of the products of either alkaline or ribonuclease hydrolysis since while (*Va*) and (*Vb*) will lead to the formation only of 3′-phosphates, (*Vc*) will give a nucleoside-3′,5′-diphosphate. Markham and Smith[44] have found no nucleoside diphosphates in the alkaline hydrolysates of the ribonucleic acids of yeast and turnip yellow mosaic virus and thus consider these acids to be represented by (*Va*) or (*Vb*). However, with the pentose nucleic acids of tobacco mosaic virus and potato virus X, Markham, Matthews and Smith[45] found diphosphates of all nucleosides and thus conclude that in these nucleic acids a large proportion of the terminal phosphate groups are on the 5′ position.

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THE SYNTHETIC POLYNUCLEOTIDES

Little has been known about the mechanisms of the synthesis of polynucleotides until recently. The discovery of an enzyme which catalyses the reversible synthesis of polyribonucleotides from the ribonucleoside-5′-diphosphates by Ochoa and co-workers, and of an enzyme catalysing the incorporation of deoxyribonucleoside-5′-triphosphates into deoxyribose nucleic acids by Kornberg and co-workers has brought about a notable advance in our knowledge of polynucleotide synthesis. Moreover, synthetic polynucleotides have been made available for study and comparison with the naturally occurring nucleic acids.

THE SYNTHETIC POLYRIBONUCLEOTIDES

The Synthesis of the Polyribonucleotides

In the course of a study of biological phosphorylation mechanisms, it was observed by Grunberg-Manago and Ochoa[1] that extracts of *Azotobacter vinelandii* catalysed a rapid exchange of ^{32}P-labelled orthophosphate with the terminal phosphate of the 5′-diphosphates (pyrophosphates) of adenosine, inosine, uridine, cytidine and, but less rapidly, guanosine. The exchange reaction, which requires the presence of magnesium ions, is accompanied by the liberation of orthophosphate. On incubation of the partially purified enzyme with inosine-5′-diphosphate in the presence of magnesium ions, Grunberg-Manago and Ochoa[2] subsequently observed that 60 to 80 per cent of the nucleoside diphosphate disappeared with the liberation of a stoichiometric amount of orthophosphate and the formation of a polymer. Alkaline hydrolysis of the polymer yielded the 2′- and 3′-inosinic acids and phosphodiesterase hydrolysed it to give 5′-inosinic acid. It was therefore concluded that the polymer was polyinosinic acid in which the nucleotides were linked through a 5′ to 3′ (or 2′) phosphodiester bond as in the pentose nucleic acids.

The reaction catalysed by the *Azotobacter vinelandii* enzyme is reversible[2], digestion of the polyinosinic acid with the enzyme in the presence of magnesium ions yielding inosine diphosphate. The reaction may thus be represented* by:

$$n(\mathrm{ppX}) \rightleftharpoons (\mathrm{pXpX})_{n/2} + n\mathrm{P}$$

where X in this case is inosine. However, the reaction is not specific for inosine and polynucleotides have also been prepared from the 5′-diphosphates of adenosine, guanosine, uridine and cytidine. Mixed polymers, using two or more different nucleoside-5′-diphosphates have also been prepared, *e.g.* polyAU and polyAGUC[3]. The reaction utilizes the energy of the pyrophosphate bond in the diphosphates to form the diester bonds of the polynucleotide chain. No accurate data have yet been obtained on the position of equilibrium, but as is the case with polysaccharide phosphorylase, it appears to be influenced by the ratio of concentration of orthophosphate to that of nucleoside diphosphate. Preliminary experiments[4] with ppA and ppI as substrate indicate that equilibrium is reached when the orthophosphate : nucleoside diphosphate ratio is about 1 : 1·5 to 2·0. The position of equilibrium at ordinary temperatures thus favours the synthesis of polynucleotide and indicates that the free energy of hydrolysis of the phosphodiester bonds in polynucleotides by the enzyme is lower than that of the pyrophosphate bonds of the nucleoside diphosphates. The ready reversibility of the reaction, however, suggests that the difference in these two free energies cannot be very great.

In view of the possibility that reversible phosphorolysis may be a major mechanism in the biological synthesis and breakdown of polynucleotides in biological systems, Grunberg-Manago and Ochoa[2] have proposed the name ‘polynucleotide phosphorylase’ for the enzyme. The enzyme appears to be widely distributed in nature[5] and polynucleotide phosphorylases have been isolated by Beers[6–10] and Olmsted[11] from extracts of *Micrococcus lysodeikticus*, by Littauer and Kornberg[12,13] from *Escherichia coli* and by Brummond, Staehelin and Ochoa[14] from a number of different bacteria.

Polynucleotide phosphorylase possesses an optimum activity[3] in the pH range 7·5 to 9·0. However its activity, and hence the rate of polymerization, is dependent not only upon the pH, but also upon the concentration of nucleoside diphosphate, magnesium ions and of other electrolytes[7,15,16]. High concentrations of potassium chloride favour the activity of the polynucleotide phosphorylase isolated from *Micrococcus lysodeikticus*, a requirement which was not evident from the enzyme obtained from *Azotobacter vinelandii*[3]. Although the presence of magnesium ions is essential for the operation of the enzyme, high concentrations of this ion are found to inhibit rather than catalyse the enzyme activity[16]. The early investigations were carried out on impure extracts of the enzyme all of which contained nucleic acid as impurity. Under such circumstances it was not possible to establish the mechanism of the enzyme reaction and in particular to determine whether a primer was necessary to start the polymerization. Mii and Ochoa[17] have obtained highly purified samples of the enzyme preparation which contain, most probably, only a single enzyme. Using this pure enzyme preparation they found that polymerization of ppA showed an autocatalytic time curve, with a long initial lag period. This lag period was eliminated by the presence of polyA, but not by polyU. In a similar way, in the polymerization of ppU the lag period was eliminated by polyU, but not by polyA.

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* The same abbreviations are used as in the previous chapter, the letters A, G, U and C refer to the ribonucleotides, with the addition of I = inosine; thus pX = nucleoside-5′-phosphate, and ppX = nucleoside-5′-diphosphate (pyrophosphate). P is used for orthophosphate. Polynucleotides will be referred to as polyA, polyC, *etc.* and mixed polynucleotides as polyAU, *etc.*

It would thus appear that the primer and nucleoside diphosphate should have a common base. However, although all polynucleotides do prime their own synthesis, they may prime, have no effect or even inhibit the synthesis of other polynucleotides. Thus, the synthesis of polyC is primed only by polyC and strongly inhibited by polyA, polyU, polyAGUC and ribonucleic acid. On the other hand, polyC primes the synthesis of polyU, polyA and polyAGUC. The mechanism of the priming effect of polynucleotides is still unknown, but the pattern of specificity observed is suggestive of a directing influence on the nature of the polynucleotide synthesized[18]. Some of these results were confirmed by Singer, Heppel and Hilmoe[19], who found in addition that certain oligonucleotides acted as primers and reduced the initial lag period; the nucleoside monophosphates, however, were not found to be primers, although oligonucleotides as small as dimers acted in this way. The oligonucleotides were found to act as true primers in as much as they were found to be incorporated into the polymer, serving as a nucleus for the polymerization, for example:

$$\mathrm{pApA} + n(\mathrm{ppU}) \xrightarrow[\text{phosphorylase}]{\text{polynucleotide}} \mathrm{pApApUpUpU}\ldots\mathrm{pU} + n\mathrm{P}$$

Evidence for this reaction was obtained by digesting the isolated polymer with ribonuclease, followed by the recovery and identification by paper electrophoresis of the adenine containing primer with one uridine residue attached, *viz.*:

$$\text{pApApUpUpU}\ldots\text{pU} \xrightarrow[\text{ribonuclease}]{\text{pancreatic}} \text{pApAUp} + (n-2)\text{Up} + \text{U}$$

A typical preparation of a polynucleotide is as follows[20]. To 100 ml. of 0·5 per cent adenosine-5′-diphosphate at pH 8·0 are added 1·7 ml. of 0·5M tris(hydroxymethyl)aminomethane to bring the pH to 9·0; 8·3 ml. of 0·01M magnesium chloride are added and then 3·3 ml. of enzyme solution. The mixture is incubated at 37°C for 280 minutes. The polymer is precipitated with concentrated potassium chloride, washed successively with 70 per cent, 95 per cent and absolute ethyl alcohol and ether. The degree of polymerization may be controlled by varying the ratio of the enzyme to substrate[21], by adding oligonucleotide primer and by varying the time of incubation.

The Nucleotide Sequence and Composition of the Polyribonucleotides

The synthetic polynucleotides consist of nucleoside-5′-phosphates linked to one another by 3′,5′-phosphodiester bonds as in natural ribonucleic acids. The chains are terminated at one end by phosphate groups monoesterified at the 5′ position of the terminal nucleoside, and at the other by a nucleotide containing free 2′ and 3′ positions. This structure is confirmed by the action of specific phosphodiesterases which act on the polynucleotides, yielding products in accordance with their action on ribonucleic acid. Thus snake venom diesterase hydrolyses the polynucleotides completely to nucleoside-5′-phosphates, while spleen phosphodiesterase yields the nucleoside-3′-phosphates and presumably a terminal nucleoside-3′,5′-diphosphate. Hydrolysis with alkali proceeds via the 2′,3′-cyclic phosphates to give mixtures of the nucleoside-2′- and 3′-phosphates. The specific action of pancreatic ribonuclease in breaking the bond between a pyrimidine nucleoside-3′-phosphate and the 5′ position of an adjacent nucleoside at the 5′ end is well brought out in the action of this enzyme on the polynucleotides. The enzyme does not attack polyA or polyI, but readily hydrolyses polyU and polyC, on exhaustive digestion, to the respective nucleoside-3′-phosphates. Shorter periods of digestion lead to the formation of oligonucleotides. Ribonuclease also hydrolyses polyAU and polyAGUC, yielding some pyrimidine mononucleotides and oligonucleotides containing all the purine and the remainder of the pyrimidine nucleotides.

The composition and type of nucleotide sequence in polyAU and polyAGUC may be determined from enzymic and alkaline hydrolysis of the polymers in the same way as described for the natural nucleic acids in Chapter 13. For a polynucleotide prepared from almost equimolecular quantities of ppA and ppU (actual ratio ppA/ppU = 1·06) and having a ratio of A : P in the polymer of 1·03, the analysis of the products obtained after exhaustive digestion with pancreatic ribonuclease is shown in *Table 30.*

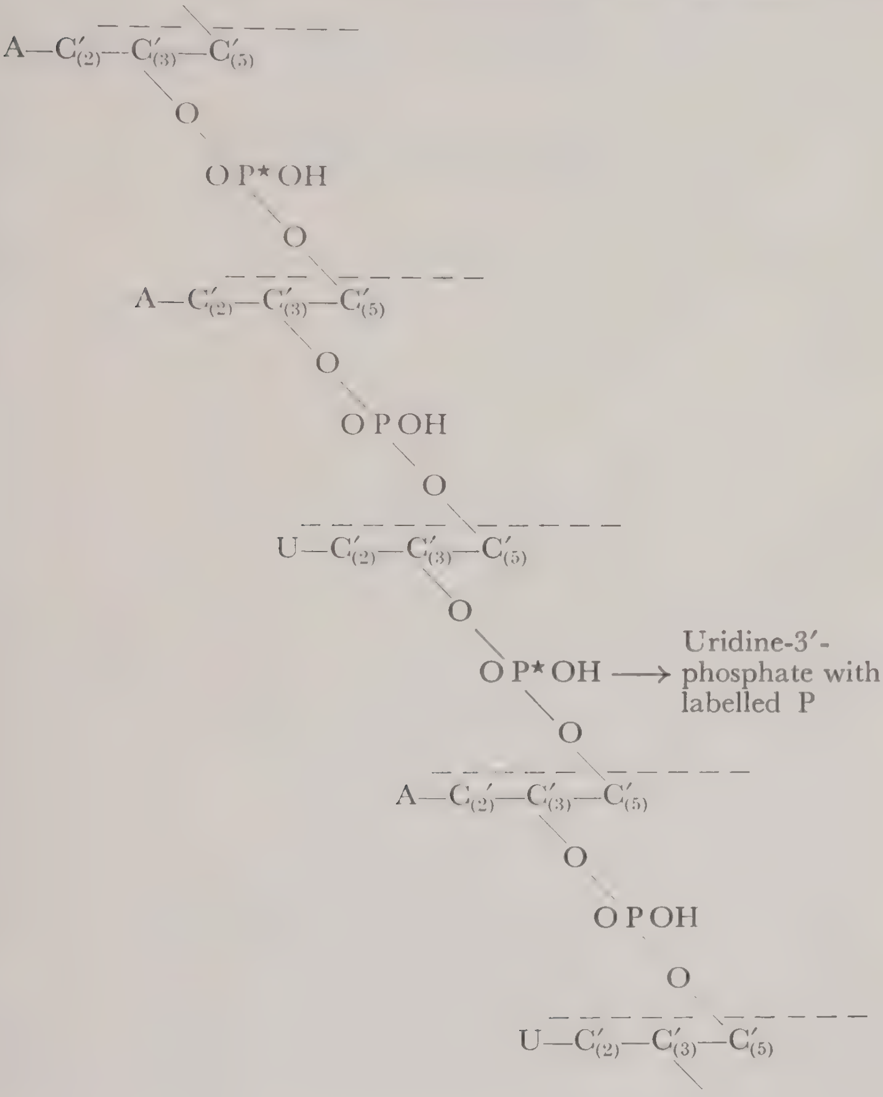
Table 30. Products of ribonuclease digestion of polyAU

Mono- or oligonucleotide	*Percentage of total products*
Up	38·0
ApUp	35·0
ApApUp	20·0
ApApApUp	4·9
ApApApApUp	2·1

(S. Ochoa and L. A. Heppel: *The Chemical Basis of Heredity* (Ed. W. D. McElroy and B. Glass): Johns Hopkins Press, Baltimore, 1957, p. 621)

Additional and confirmatory information on the sequence of nucleotides has been obtained by synthesizing the polynucleotide using adenosine-5′-diphosphate labelled with ^{32}P in the acid stable phosphate, and unlabelled uridine-5′-diphosphate[4, 5, 22]. If the polynucleotide is hydrolysed by alkali, in view of the mechanism of alkaline hydrolysis, wherever adenosine is linked to uridine, the labelled phosphorus is transferred to uridine (see (*I*), P* = ^{32}P) so that the measurement of the radioactivity appearing in uridine-3′-phosphate gives an estimate of the number of A–U links in the polynucleotide. For the polynucleotide containing a ratio of A : P of 1·03, it was found that 53 per cent of the adenylic acid residues were preceded by uridylic acid in the polynucleotide chain; the other 47 per cent must therefore have been preceded by adenylic acid. This proportion of UA to AA sequences, is very close to the theoretical value of 50 per cent and serves to emphasize the fact that the formation of polyadenylic and polyuridylic acid regions in the polynucleotide can arise as a normal consequence of random polymerization.

Further information on the sequence in polyAU may be obtained from the ribonuclease digestion of the polymer prepared from labelled adenosine-5′-diphosphate and unlabelled uridine-5′-diphosphate. The sequence pUp*ApUp*Ap will be split by ribonuclease to give ApUp*, with the radioactive phosphate group on the 3′ position of uridine, while the sequence pUp*ApUpUp will split to give ApUp with no labelled phosphorus. Thus the determination of the specific radioactivity of the dinucleotide AU released by digestion of the polynucleotide with pancreatic ribonuclease will lead to the determination of the relative frequency of the UAUA and UAUU sequences. By similar means the frequency of other sequences can be determined. In order to obtain the numbers of units occurring in various sequences, it is necessary to


A—C′(2)—C′(3)—C′(5)
O
O P* OH
O
A—C′(2)—C′(3)—C′(5)
O
O P OH
O
U—C′(2)—C′(3)—C′(5)
O
O P* OH
Uridine-3′-phosphate with labelled P
O
A—C′(2)—C′(3)—C′(5)
O
O P OH
O
U—C′(2)—C′(3)—C′(5)

(*I*)

know the average chain length. Since it has been shown that the terminal phosphate group is on the $C'_{(5)}$ and not the $C'_{(3)}$ position[3, 5], the chain length may be determined by end group analysis. Combination of the data then gives the values shown in *Table 31*. Ochoa and Heppel[4] do not consider that sufficient data have yet been obtained to warrant the detailed analysis required to determine whether the nucleotide distribution is random or otherwise.

Table 31. Nucleotide sequences in polyAU
Average chain length of polymer, 46 nucleotide residues

Sequence	*Number of residues occurring as*				*Total*
UU	UUA	7·00	UUU	9·00	16·0
UAU	UAUA	8·10	UAUU	7·10	15·2
UAAU	UAAUA	4·60	UAAUU	4·10	8·7
UAAAU	UAAAUA	1·07	UAAAUU	1·03	2·1

(S. Ochoa and L. A. Heppel: *The Chemical Basis of Heredity* (Ed. W. D. McElroy and B. Glass): Johns Hopkins Press, Baltimore, 1957, p. 624)

Ribonuclease digestion of polyAGUC follows a very similar course to that of natural ribonucleic acid. Cytidylic and uridylic acids are obtained, together with a number of oligonucleotides of which the dinucleotides, AU, AC, GU and GC and the trinucleotides AAU and AAC have been identified[4, 5, 23]. The nucleotide composition of the polyAGUC has been determined by Smellie[24] by paper chromatography and compared with the composition of the native ribonucleic acids isolated from the micro-organisms supplying the enzyme. Guanosine-5′-diphosphate does not polymerize readily[3] and for this reason, in their first preparations of polyAGUC, Grunberg-Manago, Ortiz and Ochoa used only half quantities of this nucleotide compared with the other nucleoside diphosphates, in order to increase the enzyme : guanosine diphosphate ratio. This low concentration and slow rate of polymerization clearly affected the incorporation of guanosine in the polymer as is shown in *Table 32*. In a later preparation equal ratios of the nucleotides were used and the incorporation of guanosine into the polymer was higher. Of equal significance is the low amount of uracil in the polyAGUC and in the natural ribonucleic acids (*Table 32*); it would appear that not only is the enzyme sensitive to the nucleoside diphosphate concentration, but also that it exhibits marked specificity during the polymerization.

The phosphorolysis of the synthetic polynucleotides by the polynucleotide phosphorylase has been studied by Singer[25] and by Ochoa[26]. Oligonucleotides were phosphorolysed much more slowly than the higher polymers[25] and pApA was not significantly attacked by the enzyme. The removal of the terminal 5′-phosphate group as in ApApA further reduced the rate of phosphorolysis. Of greater significance to the structure of the polymers was the observation by Ochoa[26] that while the phosphorolysis of synthetic polynucleotides containing only one kind of nucleotide (*e.g.* polyU) was rapid and complete, the phosphorolysis of polyAU, polyAGUC and of natural ribonucleic acid was very slow[3]. This was attributed to the fact that these molecules exist in solution largely as multi-stranded chains, which, contrary to the single stranded chains of polyU, are resistant to cleavage by the polynucleotide phosphorylase. This important aspect of the structure of these polymers will be discussed more fully below (p. 322).

Table 32. Nucleotide composition of polyAGUC (enzyme *Azotobacter vinelandii*) and of ribonucleic acid of *Azotobacter vinelandii*

Polynucleotide	*Adenine*	*Guanine*	*Uracil*	*Cytosine*
AGUC (1 : 0·5 : 1 : 1)	10·0	5·9	7·1	9·9
AGUC (1 : 1 : 1 : 1)*	10·0	12·7	4·2	11·1
Azotobacter ribonucleic acid	10·0	13·0	7·3	9·0

* Prepared with crude enzyme, polymer of low molecular weight.

(From S. Ochoa and L. A. Heppel: *The Chemical Basis of Heredity* (Ed. W. D. McElroy and B. Glass): Johns Hopkins Press, Baltimore, 1957, p. 626)

The Size and Shape of the Polyribonucleotides

Numerous determinations of the molecular weight of various synthetic polynucleotides have been made and the results are summarized in *Table 33*. The values are comparable with those of preparations of natural ribonucleic acids and are very close to that of the natural ribonucleic acid of *Azotobacter vinelandii*[27]. The molecular weights may also be determined from the average chain length as determined from end-group assays[33]. With polyA in neutral or weakly alkaline solution, the values of the molecular weight as determined by sedimentation and by end-group assay are in excellent agreement, but with polyAU and polyAGUC, the sedimentation molecular weights, 3 × 10^5 and 7 × 10^4 respectively, are very much greater than those obtained from end-group analysis, 4 × 10^4 and 9 × 10^3 respectively. This is attributed by Ochoa[5] to the aggregation of the mixed polymers in solution to give aggregates of high molecular weight (see below). However, there is considerable evidence that stable aggregates of polyA and polyC are also formed under certain solution conditions.

Table 33. Molecular weight of polyribonucleotides

Polyribonucleotide	*Molecular weight*	*Method*	*Reference*
Azotobacter enzymes			
A*	7 × 10^4	Sedimentation	27
A*	1·48 × 10^5	Light scattering	28
A	1·77 × 10^5	Sedimentation, diffusion	29
A	5·7 × 10^5	Light scattering	30
M. lysodeikticus enzymes			
A	1·86 × 10^6	Light scattering	10
A	1·6–2·6 × 10^6	Light scattering	20, 31
A	1·1–4·0 × 10^6	Light scattering	32
Azotobacter enzymes			
U	7 × 10^4	Sedimentation	27
U	1·73 × 10^4	Sedimentation, diffusion	29
AU	3 × 10^5	Sedimentation	27
I	8 × 10^5	Light scattering	30
AGUC	7 × 10^4	Sedimentation	27
A†	1·5 × 10^4–1·3 × 10^5	Sedimentation, viscosity	21
A‡	1·5 × 10^5–3·5 × 10^6	Sedimentation, viscosity	21

* Determined on specimens from the same preparation.
† Range of values for different preparations in which substrate concentration varied.
‡ Values refer to aggregates.

Samples of polyA, covering a wide range of molecular weights (1·5 × 10^4–1·3 × 10^5) have been prepared by Fresco and Doty[21] by varying the enzyme : substrate ratios and the time of polymerization. These samples, in solution above pH 6·5 in water or above pH 5·7 in 0·15M sodium chloride, exhibit a characteristic absorption spectrum having a maximum at 257 mμ (*Figure 81*) and gave values of the sedimentation constants and intrinsic viscosities that depended upon the molecular weights in the manner shown in the left side of *Figure 82*. The slopes of the lines correspond to those of randomly coiled chains, a result in agreement with that of Steiner and Beers[20, 31]. Below pH 5 in 0·15M sodium chloride, and below pH 6·5 in water, the polyA samples possess a different spectrum with a maximum at 252 mμ[20, 21, 34] (*Figure 81*) and have values

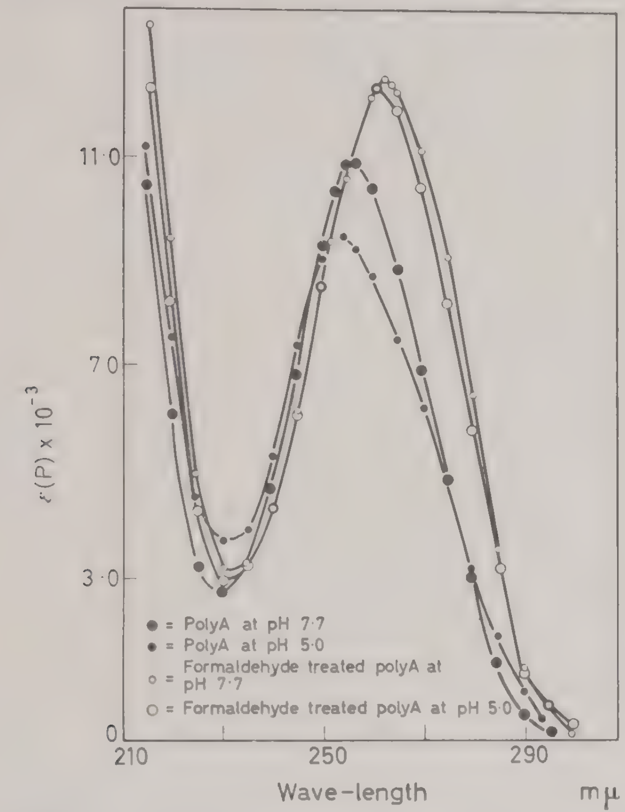


Figure 81. Ultra-violet absorption spectra for native and formaldehyde treated polyA in 0·01M potassium chloride at neutral and acid pH

(R. F. Beers and R. F. Steiner: *Nature, Lond.* 1957, **179**, p. 1077)

of the sedimentation coefficient and intrinsic viscosity which are dependent upon the concentration and temperature at which the transition was brought about by lowering the pH. By varying the concentration of polyA prior to lowering the pH to below 5·0, the sedimentation coefficient and intrinsic viscosity were found to vary with molecular weight in the manner shown on the right of *Figure 82*. The behaviour now suggests that aggregation has occurred[21, 34] and the slopes of the lines are suggestive that the species are homologous in structure and that the differences in weight are the result of different extents of linear growth (*i.e.* aggregation). Unlike the randomly coiled configuration, this form does not show a marked increase in specific viscosity[21] or of the root-mean-square end-to-end distance[20] with removal of sodium chloride. This result led Fresco and Doty[21] to conclude that the transition between the two forms is sharp. Moreover, on heating solutions of the rigid form, a transition to the random coil form occurs at about 75°C, which is very reminiscent of the heat denaturation of deoxypentose nucleic acids (see Chapter 11, p. 256).

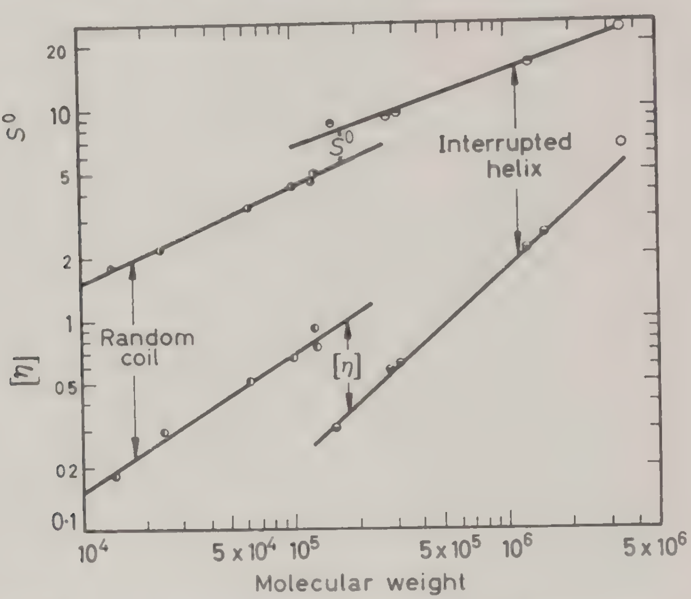


Figure 82. Molecular weight dependence of sedimentation coefficient of the two configurations of polyA

For curves on the left the solvent was 0·15M sodium chloride, 0·015M sodium citrate, pH 7·1; for those on the right the solvent was 0·15M sodium acetate, pH 4·9. Open circles represent measurements at pH 3·7 with no added electrolyte.

(J. R. Fresco and P. Doty: *J. Amer. chem. Soc.* 1957, **79**, p. 3928)

It would thus appear that polyA (and probably also polyC and polyI) can associate in solution under suitable conditions to give an aggregate. Fresco and Doty[21] believe the aggregate to be a base paired, discontinuous (since formed from smaller polynucleotides) double helix similar to that occurring in the solid state (see below). However, if this is so, the pH conditions required, *viz.* below pH 5, are rather surprising, since at this pH the adenine amino group, which would be participating in the hydrogen bonding, is being ionized to the acid charged form as would be expected from the pK_a' value of this group (4·16) and as has been shown by titration by Steiner and Beers[20]. By analogy with deoxypentose nucleic acid, it would therefore be expected that the hydrogen bonded structure was being destroyed and not formed. However, the fact that the aggregation can be prevented by the presence of formaldehyde[20, 31, 32, 34] adds confirmation to the view of Fresco and Doty that the hydrogen bonds in the double helix are of a similar type to those occurring in deoxypentose nucleic acid.

Further confirmation of the possible formation of a double helical structure in solution comes from the proposed structure of polyA in the solid, fibrous form. As will be described below, the x-ray diffraction evidence strongly suggests the presence of a double helix, stabilized by hydrogen bonds in the solid state.

Although solutions of polyI have not been studied so thoroughly as those of polyA, several features of the solution properties indicate that the polymer can exist in two forms, one an organized helical structure, and the other a disordered random single chain form[35]. The changes in solution properties are, like those of polyA, dependent on pH and ionic strength. Increase of pH brings about an increase of the extinction coefficient and a shift in the maximum from 249 mμ to 254 mμ, a shift in the same direction as with polyA, and at the same time the sedimentation constant falls from 6·75 at pH 6·8 to 2·45 at pH 10·2. Similarly, an increase of ionic strength causes changes in sedimentation coefficient and extinction coefficient indicative of an aggregation process. As will be described below, polyI in the fibrous form probably forms a triple hydrogen bonded helix. It may thus be concluded that polyI like polyA exists in solution in two forms, the single chain, random coil form and the more rigid hydrogen bonded triple helix. Confirmation of this view has recently been obtained by Doty, Boedtker, Fresco, Haselkorn and Litt[36], who observed a sharp increase in the optical density of solutions of polyI at about 42°C (*Figure 83*). Similar changes are observed on the heat denaturation of deoxypentose nucleic acid and have been likened by Doty to a melting process (see Chapter 11, p. 256). The configurational change that occurs at these critical temperatures is one from a state possessing a high degree of order to a more random one and all the evidence indicates that the change is from a hydrogen bonded helix to an intertwined random coil configuration. The temperature at which the change occurs is an indication of the strength of the bonds holding the helix together, in just the same way as the melting point of a solid is a measure of the intermolecular or interionic bond strength. The transition with polyI, 42°C, is lower than that for any other polynucleotides and lower than that for the nucleic acids; the polyI helix is therefore not a particularly stable aggregate.

The Structure of the Polynucleotides from X-ray Diffraction Studies

X-ray diffraction diagrams of polyA, polyC, polyU, polyI, polyAU and polyAGUC have been obtained. Fibres of all these polynucleotides may be drawn from concentrated solutions by a similar method to that adopted for deoxypentose nucleic acid (see Chapter 9).

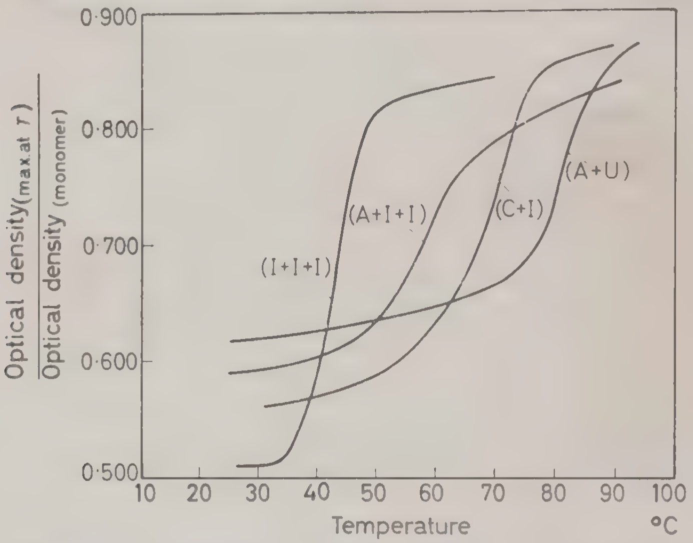


Figure 83. Variation in optical density as a function of temperature of heating of solutions of polyribonucleotide complexes

Measurements were made in 1·0M sodium chloride containing 0·002M sodium hydrogen phosphate at pH 6

(P. Doty, H. Boedtker, J. R. Fresco, R. Haselkorn and M. Litt: *Proc. nat. Acad. Sci., Wash.* 1959, **45**, p. 488)

However, the diffraction patterns produced by the polynucleotides are not identical and show the presence of marked structural differences. The diffraction patterns may be conveniently grouped into three classifications: (*1*) those showing an amorphous diffraction pattern; (*2*) those showing a diffraction pattern similar to natural ribonucleic acid; and (*3*) those showing a well defined diffraction pattern, better than that shown by natural ribonucleic acid, thus indicating a highly ordered structure. PolyU falls into the first category; fibres may be prepared readily which are not birefringent and which produce an amorphous diffraction pattern, indicating that there is virtually no repetitive structural organization

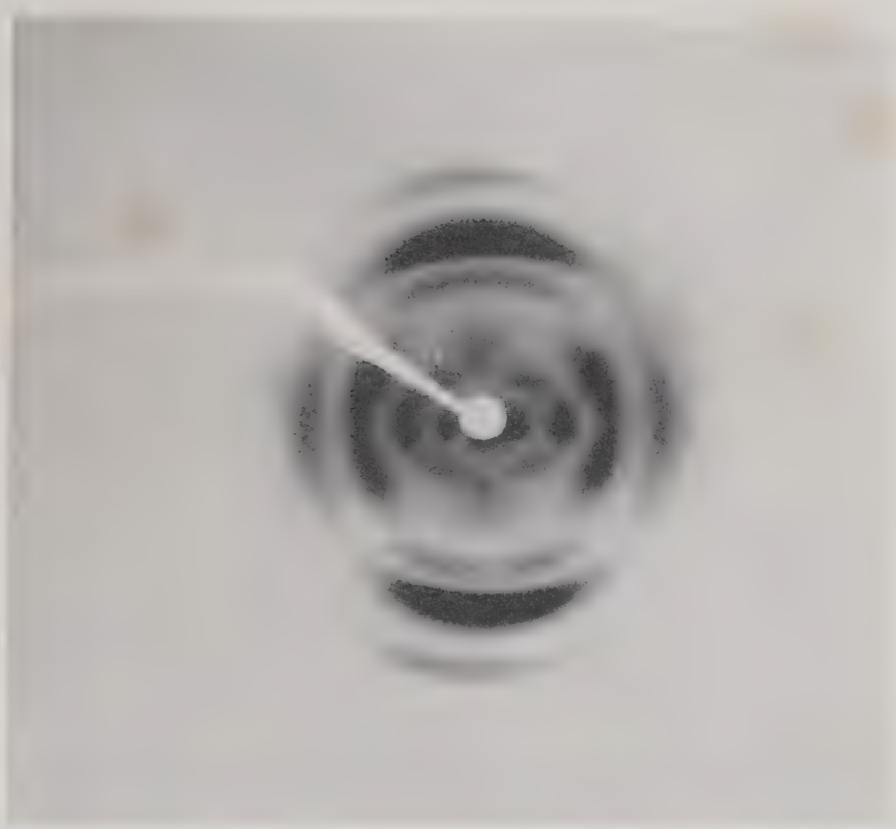


Plate 7. X-ray diffraction photograph of a polyA fibre (J. D. Watson: *The Chemical Basis of Heredity* (Ed. W. D. McElroy and B. Glass): Johns Hopkins Press, Baltimore, 1957, p. 554)

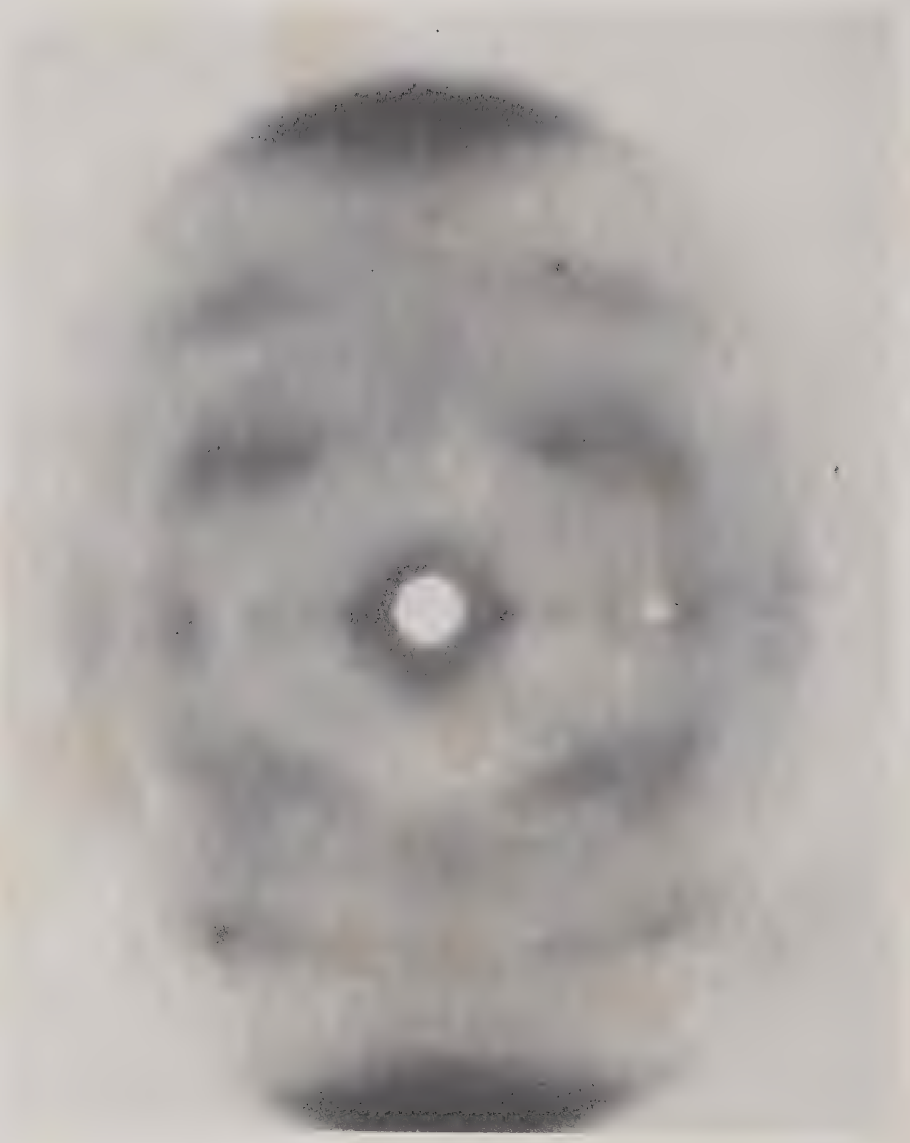


Plate 8. X-ray diffraction photograph of a polyI fibre
(A. Rich: *Biochim. biophys. Acta* 1958, **29**, p. 503)

(*To face page 325*)

in the molecule[37]. PolyAU and polyAGUC are in the second group and give diffraction patterns very similar to those of native ribonucleic acid[37-39]. The diagrams, although a little better oriented than those of ribonucleic acid, are not of sufficiently high quality to enable a direct structural interpretation to be made. Nevertheless, it is significant that both patterns are so similar, since it demonstrates that the synthetic polymers can assume a configuration similar to that of a natural nucleic acid. The last group, including polyA, polyC, and polyI, is that in which most progress has been made. All three of these polymers give x-ray photographs indicating highly ordered structures, some of the reflections being quite sharp when compared with the diffuse bands of the ribonucleic acid picture.

Detailed structural studies have been carried out on polyA[38] and on polyI[35]. The diffraction pattern of polyA (*Plate 7*) has been interpreted by Watson[38], on the basis of the relative absence of meridional reflections, as indicating a helical structure. The repeat distance along the fibre axis is at about 15 Å and the distance between the nucleotides 3·75 Å. The size of the unit cell indicates that more than one polynucleotide chain is present and of the possible two and three chain models, the two chain structure appears more probable from the value of the density. As with deoxypentose nucleic acids, on theoretical grounds there are two possible structures, one in which the two polynucleotide chains do not coil around each other, but lie side by side and the other in which the chains are intertwined on a common axis. The latter structure is found to be the more satisfactory from both a consideration of the x-ray diffraction pattern and on *a priori* structural grounds. The structure of polyA is thus considered[38] to be a double helix, with the sugar-phosphate backbone on the outside of the molecule and with the bases pointing towards the central axis, as in deoxypentose nucleic acid. Hydrogen bonds link together pairs of adenine residues on the same level, the hydrogen bonds joining the amino nitrogen in position 10 to the free nitrogen in position 7 (*Figure 84*); the adenine residues are thus oriented in a similar manner to that found in crystals of adenine hydrochloride[40]. Only one of the hydrogen atoms of the amino groups is involved in the base pairing and it is possible that a second hydrogen bond will form with the non-esterified oxygen atom in the phosphate group of the opposing chain. Although this model satisfies many of the requirements of the x-ray data, it is still somewhat tentative.

The x-ray diffraction pattern of polyI (*Plate 8*) shows many unusual features when compared with that of either deoxypentose nucleic acid or any of the other polynucleotides[35]. There are three layer lines, two are non-meridional and occur at spacings of 9·8 Å and 5·2 Å, while the third is a very strong meridional reflection at 3·4 Å. This latter reflection will be associated with the stacking of the planar purine rings at right angles to the fibre axis, as in many other natural and synthetic polynucleotides. The interpretation given to the x-ray diffraction diagram by Rich[35], which is based on considerations of the screw rotation between successive residues, is that the polyI fibre consists of a three strand structure in which the hypoxanthine residues are linked by hydrogen bonds as shown in *Figure 85*. Rich[35] considers that a two stranded model must be ruled out since the sugar-phosphate backbone cannot be extended sufficiently to permit the screw rotation angle between successive nucleotides of 62·5° which would be necessary. It is not clear why such a structure can be made for polyA (see above) and not for the very similar polymer, polyI, the only structural difference being the different nature of the hydrogen bonds. Although a four strand model is possible, it is ruled out on the grounds that such a molecule would contain a central hole, about 2·3 Å in diameter, which would introduce considerable molecular instability.

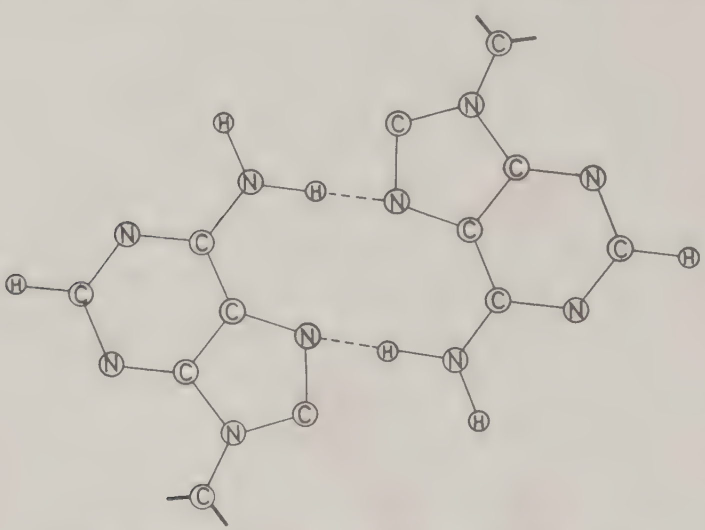


Figure 84. Hydrogen bonds linking pairs of adenine residues in polyA

It is thus evident that on the basis of the x-ray diffraction diagrams, some synthetic polynucleotides are capable of assuming configurations not unlike the ordered structures observed in the natural nucleic acids. Thus the classification of the x-ray diffraction pattern given above may now be explained in structural terms. Those polynucleotides giving amorphous patterns, such as polyU, exist as single strand, random coil structures, while those in groups (*2*) and (*3*), showing better defined diffraction patterns, possess helical hydrogen bonded structures containing two or three polynucleotide chains. The interpretation of the x-ray data is in agreement with the conclusions drawn from the changes in size and shape occurring in solution and in particular is in marked conformity with the ultra-violet absorption measurements of Warner[41]. The extinction coefficient [ϵ(P)] of polyA at 258 mμ at pH 7 in the absence of salt is 61 per cent of that of adenylic acid, while that of polyU is 95 per cent of that for uridylic acid. Increase of the ionic strength of solutions of polyA raises the value of ϵ(P) to 61 per cent of the adenylic acid value, while 6·4M urea increases it still further to 87 per cent of this value. Change of ionic strength and the addition of urea has no effect on the value of ϵ(P) of polyU. PolyC, polyG, and polyI also show abnormal suppressions of ϵ(P) compared with the value for the respective nucleotides. Such hyperchromic effects are in conformity with the hydrogen bonded helical structure

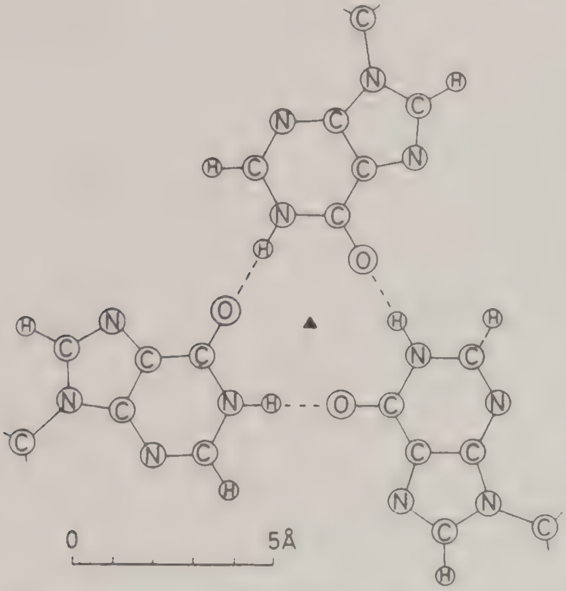


Figure 85. **The hydrogen bonding system for the three stranded form of polyI. Three hypoxanthine bases are arranged about a three-fold rotation axis held together by hydrogen bonds.**

(A. Rich: *Biochim. biophys. Acta* 1958, **29**, p. 505) of polyA and polyI, where interaction of the purine groups brings about the suppression of $\epsilon(P)$ and with the single chain, random coil structure of polyU where such interaction cannot occur.

The Interaction between Polynucleotides

While studying the properties of polyAU and comparing its properties with those of a mixture of polyA and polyU to ascertain whether it was a true polymer or not, Warner[41] observed that a specific interaction occurred between these two polymers so that the $\epsilon(P)$ value of the mixture was 68 per cent of that calculated for a mixture of the polymers and 55 per cent of that for the constituent mononucleotides. No other pair of polynucleotides showed this marked reduction of $\epsilon(P)$, although Rich[42] has since shown that polyA and polyI also react together, and Davies and Rich[43] that polyI and polyC form an aggregate. The occurrence of an aggregation of the two polymers was established by electrophoresis and sedimentation studies[44]. The conditions for electrophoresis of the polymers were arranged so that a substantial difference in mobility existed between polyA and polyU. At pH 9·6 at 0°C and an ionic strength of 0·1, polyA has a mobility of $10{\cdot}6 \times 10^{-5}$ cm^2 V^{-1} sec^{-1} and polyU, $13{\cdot}2 \times 10^{-5}$. This difference is sufficient to permit a resolution of the two polymers to be made if no interaction occurred. However, when a mixture of the two polymers was subjected to electrophoresis in the same buffer, a single peak was observed having a mobility of $11{\cdot}1 \times 10^{-5}$. A corresponding result is obtained on sedimenting mixtures of these polymers. The data of several observers are summarized in *Table 34*. The mixture of polynucleotides is found always to sediment as a single boundary, with a sedimentation coefficient far greater than that of either of the polynucleotides separately and this is reflected in the one instance in which the molecular weights have been determined, by the very much greater molecular weight of the aggregate compared with that of the constituent nucleotides.

Further evidence supporting the specific interaction between these pairs of polymers comes from the observation of Ochoa[26] that while the phosphorolysis of polyU by polynucleotide phosphorylase occurs rapidly and is almost complete in 1 hour, that of poly(A + U) is very much slower and incomplete and after 5 hours only 20 per cent of the reaction is complete. This would indicate that the incorporation of polyU in the complex has markedly affected its activity towards the enzyme. It is also noteworthy that polyA, which complexes in solution, although to a lesser extent than poly(A + U), also reacts more slowly than polyU which remains as a non-hydrogen bonded random coil in solution.

The interaction between the two polynucleotides also produces a marked increase in the viscosity of the solution[46] and a change in the complete absorption spectrum as well as the depression of the maximum referred to above[42] (*Figure 86*). From studies of the specific interaction of polynucleotides in solution by Felsenfield, Davies and Rich[42, 45, 46] and Warner[44] the main features of the reaction have been obtained. A convenient method for determining the molar ratios of the individual polynucleotides in the aggregate

Table 34. Sedimentation coefficient, diffusion coefficient and molecular weight of polyribonucleotides and mixed aggregates

Polyribo-nucleotide	*Conc.* Molarity	pH	*I*	S_{20} S units	D_{20} cm²/sec × 10^7	*M*	*Refer-ence*
PolyA	—	7	—	2·5	—	8×10^4†	43
PolyU	—	7	—	2·2	—	8×10^4†	
Poly(A+U)*	—	7	—	5·3	—	—	
PolyA	0	—	—	5·3	1·7‡	$1{\cdot}77\times10^5$§	43
PolyU	0	—	—	2·2	7·4‡	$1{\cdot}73\times10^4$§	
Poly(A+U)	0	—	—	9·9	1·3‡	$4{\cdot}5\times10^5$§	
PolyA	—	—	—	8·0	—	—	44
PolyU	—	—	—	3·2	—	—	
Poly(A+U) (1 : 1)	—	—	—	12·6	—	—	
Poly(A+U) (1 : 2)	—	—	—	17·3	—	—	
PolyA	6×10^{-5}	6·8	0·05	7·2	—	—	41
PolyI	6×10^{-5}	6·8	0·05	6·8	—	—	
Poly(A+I) (1 : 1)	6×10^{-5}	6·8	0·05	10·4	—	—	
Poly(A+I) (1 : 2)	6×10^{-5}	6·8	0·05	14·7	—	—	
PolyI	—	6·7	0·1	7·4	—	—	42
PolyC	—	6·7	0·1	5·5	—	—	
Poly(I+C)	—	6·7	0·1	13·9	—	—	

* The mixed aggregate is summarized as poly(A + U), *etc.*; not to be confused with polyAU, *etc.* which refers to the co-polymer.

† Determined by end-group analysis.

‡ Concentration 0·2 per cent.

§ Partial specific volume 0·58.

formed has been developed by Felsenfield and Rich[47, 48]. This makes use of the variation of the optical density of mixtures with the composition. If the sum of the concentrations of polyA and polyU is kept constant and the optical density determined at different molar ratios, it can quite easily be shown that two straight lines will be obtained which intersect at the molar ratio of the complex. The method is illustrated in *Figure 87* where the optical densities at 259 mμ of mixtures of polyA and polyU in 0·1M sodium chloride are given as a function of the mole fraction. The formation of a 1 : 1 complex is clearly indicated. The formation and the rate of formation of the complexes are very dependent upon both the ionic strength and the nature of the ions present. In distilled water solution no complex is formed[44, 47] as shown by the complete absence of a hyperchromic effect; the addition of ions, however, brings about a decrease in the optical density and the formation of the complex as shown in *Figure 88*. This behaviour is reversible[47] and when considered in terms of the removal of ions to cause an

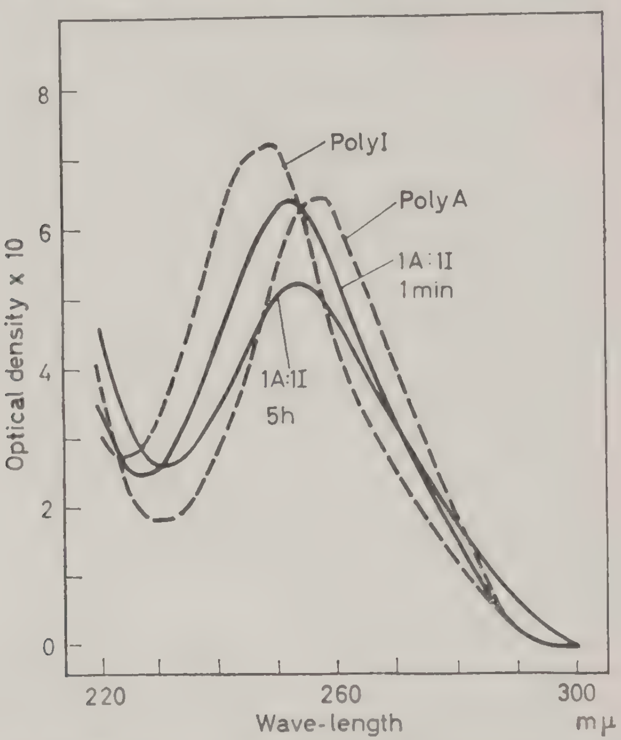


Figure 86. The change in absorption spectra of polynucleotides produced by interaction between two different polynucleotides (A. Rich: *Nature, Lond.* 1958, **181**, p. 522)

increase in the optical density has similarities to the denaturation of deoxypentose nucleic acid on dilution of the electrolyte, the main difference being the irreversibility of the latter process (see Chapter 11, p. 242).

In the presence of 10^{-2}M magnesium or other divalent cations or high concentrations of sodium chloride (0·7M), Felsenfield, Davies and Rich[45, 47] found that the mixing curve assumes a new shape (*Figure 89*), in which the minimum occurs at a molar ratio of uridine to adenine of 2 : 1. Rich[42] has observed similar behaviour for complexes formed between polyI and polyA, although in this case the 2 : 1 complex is formed more readily. In 0·05M sodium chloride the 1 : 1 complex is obtained immediately on mixing, but the optical density changes with time and 5 hours after mixing the formation of the 2 : 1 complex is complete. In view of the dependence on the ionic environment it would appear that the formation of the complexes is very dependent on the exact ionic state of the participating polynucleotides. If solutions of the mixed polynucleotides are heated a sharp rise in optical density occurs[44]

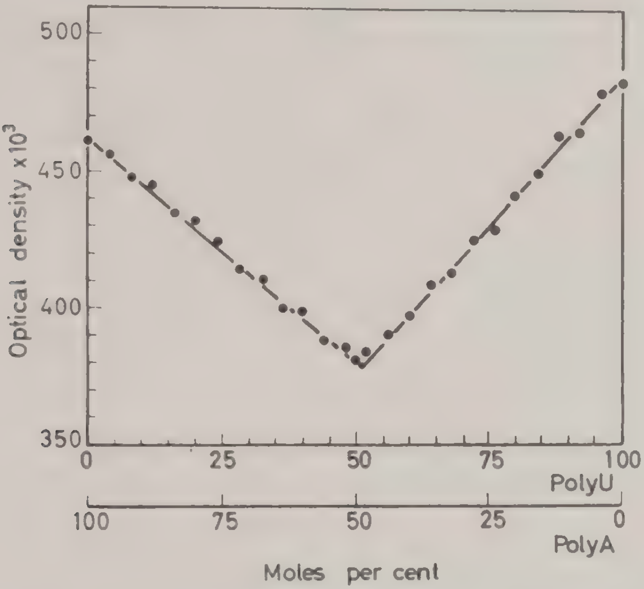


Figure 87. The variation of optical density at 259 mμ of mixtures of polyA and polyU as a function of composition. The optical densities were measured within 15 minutes of mixing. All solutions were in 0·1M sodium chloride, 0·01M glycyl–glycine, pH 7·4.

(G. Felsenfield and A. Rich: *Biochim. biophys. Acta* 1957, 26, p. 461) at a temperature which is characteristic for each mixed polymer[36] (*Figure 83*). The hydrogen bonded complex thus dissociates (denatures) into the individual polynucleotides which will assume an intertwined random coil configuration. This process is reversible, since the optical density falls again on cooling. This process is also similar to the heat denaturation of deoxypentose nucleic acid, except for the important difference that in the latter case it is irreversible. The temperature at which the transition occurs indicates that the stability of the complexes increases in the order poly(A + 2I), poly(C + I), poly(A + U).

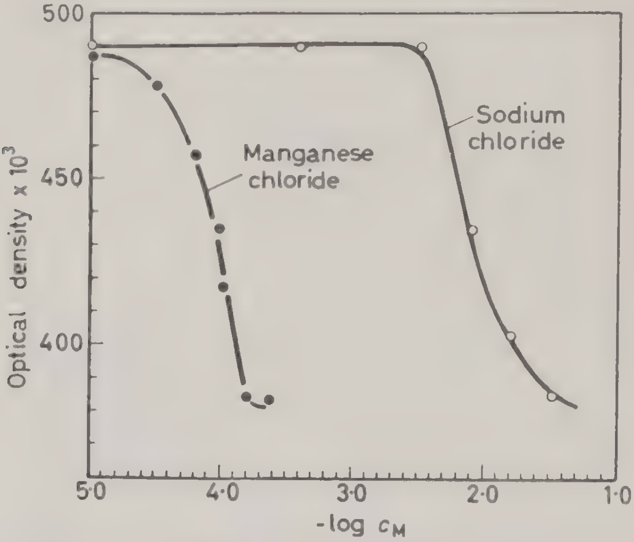


Figure 88. The effect of the addition of metal ions on the optical density of a 1 : 1, A : U mixture in distilled water. The optical density is plotted against the total concentration.

(G. Felsenfield and A. Rich: *Biochim. biophys. Acta*, 1957, **26**, p. 462)

From the concentrated, viscous solutions of the complexes, tough glassy fibres can be drawn, which are negatively birefringent[46]. With these fibres, well defined x-ray diffraction patterns have been obtained. That for poly(A + U) suggests[46] that the molecule is a two stranded helix containing one strand of polyA and one of polyU. Hydrogen bonds occur between adenine and uracil in the same manner as that postulated for adenine and thymine in deoxypentose nucleic acid (see Chapter 9, p. 161) with the base pairs stacked one above the other roughly perpendicular to the fibre axis. The two sugar-phosphate backbones have a smaller angular separation when viewed from the helical axis than occurs in the deoxypentose nucleic acid molecule. The most simple explanation of this is obtained by assuming that the backbone chains have a parallel orientation instead of the anti-parallel orientation postulated for deoxypentose nucleic acid (see Chapter 9, p. 160). A similar structure has been proposed by Rich[42] for the structure of poly(A + I) from the interpretation of the x-ray diffraction pattern obtained with fibres of this polymer. The hydrogen bonding between hypoxanthine and adenine which links the two intertwined helical chains is shown in *Figure 90*. The amino group of adenine is linked to the keto-oxygen of hypoxanthine, while both purine nitrogen atoms are linked by a hydrogen bond. The distance between the stacked hydrogen bonded purines is 3·4 Å.

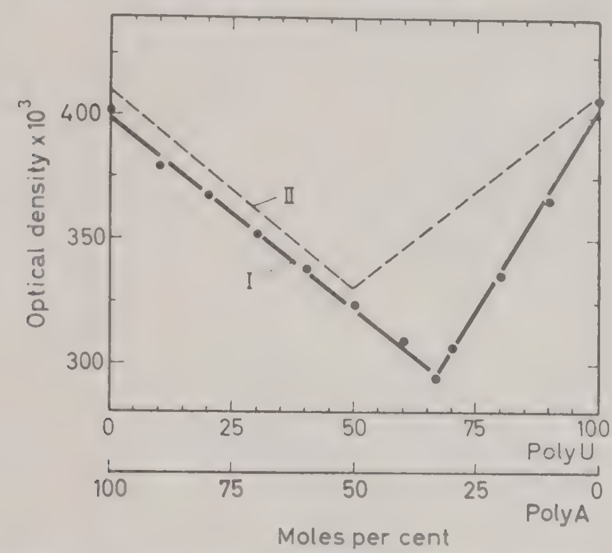


Figure 89. The optical density of various mixtures of polyA and polyU
I, solutions containing 0·1M sodium chloride, 0·01M glycyl-glycine (pH 7·4) and $1{\cdot}2 \times 10^{-2}$M magnesium chloride; II, the optical density before the addition of magnesium chloride
(G. Felsenfield and A. Rich: *Biochim. biophys. Acta* 1957, **26**, p. 462)

No x-ray studies have been made, as yet, on the complexes poly(A + 2U) and poly(A + 2I). However, in both cases Rich[42] and Felsenfield, Davies and Rich[45] consider from the spectroscopic and sedimentation data that the complexes must have a three stranded structure. On the basis of the two stranded models for poly(A + U) and poly(A + I), it is considered that the third strands of polyU and polyI respectively lie in the helical groove of the two stranded molecule. Rich[42] suggests, for example, that in poly(A + 2I), the second hypoxanthine base is linked to the adenine residue by two hydrogen bonds, the hypoxanthine keto-oxygen and $N_{(1)}$ being bonded respectively to the amino-nitrogen and $N_{(7)}$ of adenine. A similar arrangement has been suggested for

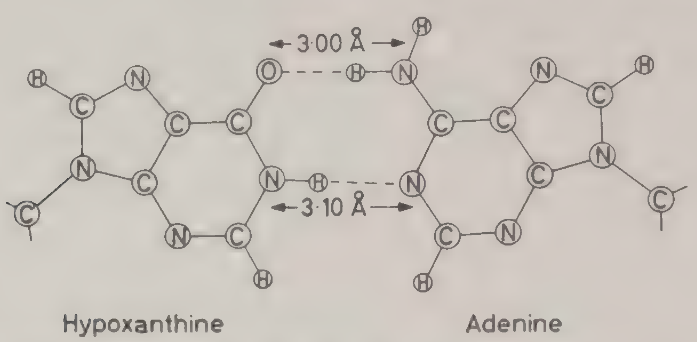

3·00 Å
3·10 Å
Hypoxanthine
Adenine

Figure 90. Hydrogen bonding between hypoxanthine and adenine in poly(A + I)
(A. Rich: *Nature, Lond.* 1958, **181**, p. 524)

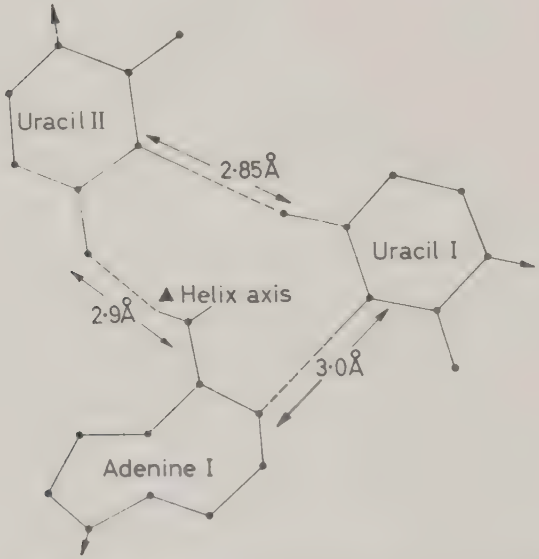

Uracil II
2·85Å
Uracil I
2·9Å
▲ Helix axis
3·0Å
Adenine I

Figure 91. Hydrogen bonding between two uracil and one adenine residue in poly(A + 2U)
The plane of adenine I is tilted about 44° relative to uracil I and about 37° relative to uracil II
(G. Zubay: *Nature, Lond.* 1958, **181**, p. 389)

poly(A + 2U)[45]. This structure has been criticized by Zubay[49] on the grounds that magnesium ions are chelated as part of the complex and probably occupy some of the sites suggested above as involved in hydrogen bonding. If this were so, hydrogen bonds involving these sites would not be possible and Zubay[49] has suggested an alternative three stranded structure (*Figure 91*) for poly(A + 2U). This structure involves three intertwined polynucleotide strands equivalently situated in the helix, in contrast to the model of Rich in which the third strand occupies a groove in the double helix structure. This means that in the reaction poly(A + U) + U → poly(A + 2U) a molecular rearrangement of the first complex must take place. Preliminary x-ray diffraction diagrams of poly(A+2U) are regarded as confirming in a general way the main features of this structure[49].

THE SYNTHETIC POLYDEOXYRIBONUCLEOTIDES

Following the discovery by Kornberg, Lehman and Simms[50] that thymidine (labelled with ^{14}C in the $C'_{(2)}$ position) was converted by enzyme fractions from normal *Escherichia coli* into a polydeoxyribonucleotide, the synthesis of high molecular weight polydeoxyribonucleotides has been developed by Kornberg and his co-workers. The sequence of reactions for the incorporation of thymidine in the polydeoxyribonucleotide was found to be:

$$\text{thymidine} \rightarrow \text{thymidine-5'-phosphate} \rightarrow \rightarrow \text{thymidine triphosphate} \rightarrow \text{polymer}$$

The discovery that the thymidine was first converted into the -5′-triphosphate before polymerization occurred, led Kornberg, Lehman, Bessman and Simms[51–53] to use the 5′-triphosphates of all deoxyribonucleotides (labelled with ^{32}P on the first phosphate group) which had been prepared enzymically, in the preparation of the polymers. For the synthesis to occur it was found necessary to have present magnesium ions and a primer in the form of deoxyribose nucleic acid. The polymerization by the enzyme, named ‘ polymerase ’[54], was found to occur only if all four deoxyribonucleoside triphosphates were present, the omission of any one of them from the substrate reduced the rate to about 0·5 per cent of that of the complete system[52, 55]. The reaction is reversible and pyrophosphate is produced along with the polydeoxyribonucleotide, the latter containing all the radioactive phosphorus. The reaction can be formulated as follows*:

---

*The letters, T, G, C and A refer here to deoxyribonucleosides; DNA = deoxyribose nucleic acid.

$$\begin{bmatrix} \text{ppp}^{\star}\text{T} \\ \text{ppp}^{\star}\text{G} \\ \text{ppp}^{\star}\text{C} \\ \text{ppp}^{\star}\text{A} \end{bmatrix}_n + \text{primer (DNA)} + \text{polymerase enzyme} + \text{Mg}^{2+}$$

$$\rightleftharpoons \text{polydeoxyribonucleotide} + 4n\text{PP}$$

Extensive purification of the enzyme[54] has not succeeded in completely freeing it of deoxyribonuclease; the polymers obtained therefore may not have maximum molecular weights.

The need for the presence of all four deoxyribonucleoside triphosphates and of deoxyribose nucleic acid along with the enzyme if significant amounts of deoxyribose nucleic acid are to be synthesized, suggests that the added nucleic acid is acting as a primer and template[55] and the extensive synthesis of polydeoxyribonucleotides is only possible when all the complementary deoxyribonucleosides are available for polymerization. Replacement of the deoxyribonucleoside-5′-triphosphates with ribonucleoside-5′-triphosphates prevents the polymerization; for example, the replacement of deoxyadenosine-5′-triphosphate by adenosine triphosphate in the substrate prevented the formation of polymer[55]. However, the replacement of thymine by uracil by using deoxyuridine-5′-phosphate in place of thymidine-5′-phosphate did not inhibit the reaction[56] and uracil was incorporated in the polydeoxyribonucleotide in place of thymine. In the same way it was found possible to replace thymine by 5-bromouracil, cytosine by 5-methyl- and 5-bromocytosine, and guanine by hypoxanthine but not by xanthine. Although hypoxanthine lacks the amino group on the 2 position that exists in guanine, the other two substituents responsible for the hydrogen bonding of guanine to cytosine remain. The lower observed rate of incorporation of deoxyinosine triphosphate compared with deoxyguanosine triphosphate may be due to the absence of the third hydrogen bond between the guanine amino and the keto group of cytosine. There appears to be no explanation at present for the failure to incorporate deoxyxanthine triphosphate in the polydeoxynucleotide[57].

The form of the deoxypentose nucleic acid employed as the enzyme ‘ primer ’ is important, since nucleic acid treated with acid or deoxyribonuclease will not promote the reaction. Native undenatured, and also carefully denatured deoxypentose nucleic acid from several sources can be used for the purpose. The synthesized deoxypolynucleotide has been shown[55] by enzymic hydrolysis to have the typical 3′-5′-phosphodiester bonds of nucleic acids.

The first stage in the synthesis, *i.e.* the addition of a single deoxynucleotide to the primary deoxypentose nucleic acid, has been studied by Adler, Lehman, Bessman, Simms and Kornberg[58], by reacting only one of the four possible deoxynucleoside triphosphates (labelled with either ^{14}C or ^{32}P) with deoxypentose nucleic acid, the concentration of the latter being increased twenty-five fold over that normally used for priming the reaction in order to increase the yield. Although it is not possible to be definite owing to the polydispersity of the deoxyribose nucleic acid and the lack of information about the size and number of deoxyribose nucleic acid molecules in the sample that are capable of acting as primer, the the results indicate that, on average, each single deoxypentose nucleic acid molecule adds on one deoxyribonucleotide. The reaction cannot proceed further owing to the absence of the other three deoxyribonucleoside triphosphates necessary for the synthesis of a polydeoxynucleotide. Furthermore, by analysis of the products of enzymic digestion it was established that the single deoxynucleoside triphosphate formed a 3′,5′-phosphodiester bond with the deoxynucleoside end of the deoxyribose nucleic acid molecule.

In view of the fact that the enzyme promotes the addition of a deoxynucleotide at the end of the priming molecule of deoxypentose nucleic acid, it would be expected, because of the base pairing in the specifically hydrogen bonded structure, that in the synthetic polydeoxynucleotide, (*1*) the ratios of adenine to thymine and of guanine to cytosine would both be unity, and (*2*) the base analysis would be the same as that of the primer. These expectations have been shown to be correct by Lehman, Zimmerman, Adler, Bessman, Simms and Kornberg[59], as shown in *Table 35*. It would thus appear that the priming deoxypentose nucleic acid provides the template and the polydeoxynucleotide produced is a true nucleic acid equivalent in all respects to the native preparation. A critical test of this replication hypothesis would be the reproduction of transforming activity inherent in certain deoxypentose nucleic acids. Unfortunately, so far experiments have proved inconclusive[59], due most probably to the presence in the enzyme of deoxyribonuclease activity which causes some degradation of the product.

Although in general the polydeoxynucleotides are formed only in the presence of all four deoxynucleoside triphosphates and priming deoxyribose nucleic acid, a co-polymer of deoxyadenylic acid and thymidylic acid is formed by the polymerase in the absence of added deoxyribose nucleic acid after a lag period of 3 to 6 hours. This co-polymer initiates the synthesis of new polymer without any time lag, which even when all four deoxynucleoside triphosphates are present in the substrate contains only deoxyadenylic acid and thymidylic acid. This synthesis further substantiates the replication function of the enzymic synthesis. So far no polymer has been prepared containing only deoxyguanylic and deoxycytidylic acids.

Table 35. Purine and pyrimidine composition of enzymically synthesized polydeoxyribonucleotides

Source of deoxypentose nucleic acid primer	A	T	G	C	$\frac{A + T}{G + C}$	$\frac{A + G}{T + C}$
M. phlei						
Primer	0·65	0·66	1·35	1·34	0·49	1·01
Product	0·66	0·80	1·17	1·34	0·59	0·85
A. aerogenes						
Primer	0·90	0·90	1·10	1·10	0·82	1·00
Product	1·02	1·00	0·97	1·01	1·03	0·99
E. coli						
Primer	1·00	0·97	0·98	1·05	0·97	0·98
Product	1·04	1·00	0·97	0·98	1·02	1·01
Calf thymus						
Primer	1·14	1·05	0·90	0·85	1·25	1·05
Product	1·19	1·19	0·81	0·83	1·46	0·99
T2 bacteriophage						
Primer	1·31	1·32	0·67	0·70	1·92	0·98
Product	1·33	1·29	0·69	0·70	1·90	1·02

(From I. R. Lehman, S. B. Zimmerman, J. Alder, M. J. Bessman, E. S. Simms and A. Kornberg: *Proc. nat. Acad. Sci. Wash.* 1958, **44**, p. 1193)

Up to the present few physico-chemical studies have been made on the polydeoxynucleotides. Ultracentrifugal analysis[60] has revealed that the material is polydisperse, with sedimentation coefficients ranging from 20S to 30S in different preparations as compared with 25S for the deoxyribose nucleic acid used as primer. The intrinsic viscosity of the synthetic polymer is between 1·5 and $3{\cdot}0 \times 10^3$ c.g.s. units compared with $5{\cdot}0 \times 10^3$ for calf thymus deoxyribose nucleic acid. From these data the molecular weight of the synthetic polydeoxyribonucleotide is found to be about 5×10^6. Heat denaturation at 100°C reduces the intrinsic viscosity markedly to $0{\cdot}1 \times 10^3$ c.g.s. units, although the sedimentation coefficient reduces only to 14S. This is in conformity with the behaviour of native deoxyribose nucleic acid and there is every indication from the preliminary study that the synthetic polymer in solution is best represented by the stiff coil model. The copolymer containing only deoxyadenylic and thymidylic acids, as has been described in Chapter 11 (p. 263) denatures at a temperature of 69°C which is in conformity with the variation of denaturation temperature with composition of native deoxyribose nucleic acid[61].

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15

THE HELICAL STRUCTURE AND REPLICATION OF NUCLEIC ACIDS

From the evidence presented in the previous chapters on the structure of the nucleic acids, it would appear well established that these substances, both in the pentose as well as in the deoxypentose series, possess a coiled helical structure stabilized by hydrogen bonds. The observation that the synthetic polynucleotides and some synthetic polypeptides may also take up a helical configuration in solution under suitable ionic conditions raises the question whether the hydrogen bonded, helical structure is the true form of the nucleic acids as existing in the cell or virus, since it is not inconceivable that such a structure could have been produced, as an artifact, during the isolation procedure. Direct evidence on this point will require *in vitro* studies of nucleic acids which will involve many difficulties. Not only will there be technical difficulties to be overcome in order to study the structure of a macromolecule in its native environment, but since the nucleic acids do not exist in the cell except in combination with protein it will be necessary to know much more about the structure of nucleoproteins than is known at present.

X-ray diffraction studies on fixed and unfixed cells and nuclei have been made by Wilkins[1]. Living cells are rapidly killed when exposed to x-rays, but Wilkins considers that it should be possible to obtain x-ray diffraction photographs of nucleoprotein in living cells by passing a stream of cells through the diffraction camera so that each cell receives a dose of x-rays less than the fatal dose. Although such studies have yet to be made, an x-ray photograph of the unfixed sperm of *Loligo* in an intact spermatophore and living at the beginning of the exposure has been obtained. The diffraction pattern was very similar to that obtained using sperm which had been removed from the chromatophore, washed in water, dried and then rehumidified. Furthermore, the diffraction pattern had marked similarities to that of purified deoxypentose nucleic acid. This important experiment gives direct evidence that there appears to be little or no major change in the structure of deoxypentose nucleic acid on extraction, purification and drying. Further, strong circumstantial evidence arises from the transforming activity of some deoxypentose nucleic acids and the infectivity of virus ribonucleic acids, which, being biologically active, must possess the true native configuration and which appear to have the same properties and structure as other nucleic acids. The irreversible character of the denaturation of deoxypentose nucleic acid shows that the specific hydrogen bonded structure cannot be formed under conditions which lead to the aggregation of the polynucleotides and is therefore unlikely to have been formed during the isolation procedure.

The above evidence lends support to the view that the hydrogen bonded helical structure is not an artifact but represents the true structure of nucleic acids in the cell. There is, however, the contrary evidence that while the ribonucleic acid in tobacco mosaic virus is considered to exist as a single strand helix, the extracted ribonucleic acid is, from the present interpretation of the x-ray diffraction diagram, a twin stranded helix (see Chapter 12). However, this evidence for a possible aggregation during extraction applies only to the ribonucleic acids, of which our knowledge of structure is at present much less definite than of the deoxypentose nucleic acids. Therefore, although the possibility of aggregation or some other structural change during isolation (by mild procedures) must not be completely overlooked, it would appear to be improbable for all nucleic acids and most improbable for the deoxypentose nucleic acids.

The studies of the formation, in solution, of hydrogen bonded helices by the synthetic polynucleotides, show a marked dependence of the stability of the resulting helix on the specificity of the hydrogen bonds. Thus the stability appears to follow the order polyA, poly(A + U), polyAGUC, ribonucleic acid. The stability of the last two members of this series appears to be comparable. The stability thus increases with the complexity which in turn will affect the specificity. In all probability the hydrogen bonds in polyA will not involve all the adenine residues but only sufficient to maintain the two strands as a single hydrodynamic unit. Such bonding is not only relatively weak, but reversible and is to be likened to that which occurs in denatured deoxypentose nucleic acid. The denaturation of deoxypentose nucleic acid breaks the specific hydrogen bonds which produce the unique structure and this process is irreversible, but some reversible and weak hydrogen bonding occurs in the denatured unit. In polyA, although there is strong evidence for aggregation to a more rigid unit, the formation of a complete helical configuration is still speculative although helical regions within the aggregate may well be formed.

The variation of the stability of the hydrogen bonded nucleic acid structure, as shown by the temperature at which heat denaturation occurs (see Chapter 11) adds an additional parameter to the list of possible differences between nucleic acids to which differences of genetical behaviour can be attributed. It has already been shown that differences between nucleic acids can arise from differences of composition, sequence and molecular weight, and to these we can now add stability. This will not be an entirely independent variable as it will be related to and determined by all the others. However, in view of the suggested replication mechanisms, in which separation of the polynucleotide strands is a necessary stage (see below) the energy requirements of the replication procedure will, in part, be determined by the stability of the helical structure.

The replication mechanism has been the subject of much discussion and speculation[2]. If it is fully substantiated that twin stranded helices, or even twin stranded helical regions, are formed by synthetic polynucleotides in solution, either between like or different polynucleotides, the complete study of such reactions will be most important to our understanding of the replication mechanism of nucleic acids. All the evidence both for the native nucleic acids and the synthetic polynucleotides indicates that the two strands are intertwined on a common axis, *i.e.* the molecule is formed by plectonemic and not paranemic coiling. Replication, as originally envisaged by Watson and Crick[3], must thus involve the unwinding of the helix, if each chain is to serve as the template for the formation of its complement. This means that for each turn to be unwound, the whole double helix has to be rotated one complete turn around its axis. Levinthal and Crane[4] have calculated the unwinding energy and found it to be only 150 cal/mole of replicated chain link, a value which is small compared with the total energy required for the formation of the phosphodiester linkage in the polynucleotide chain. The actual shape of the deoxypentose nucleic acid molecule will not be straight, but bent, folded or coiled. Levinthal and Crane, however, consider that it will rotate like a speedometer cable without any off-axis motions. It is generally assumed that the replication process occurs simultaneously with the untwisting, so that at any given moment a Y-shaped configuration exists as in *Figure 92*, all three arms of the Y rotating in the same direction. More complex methods of untwisting have been suggested, but the energies involved will not be very different from the mechanism originally suggested.

Other, quite different, methods of replication which have been suggested include the enzymic digestion of one polynucleotide chain, leaving the other free to serve as a template and the separation of the two complementary chains by a series of breaks and reunions of the chains. Delbrück[5] considers that these breaks and reunions occur *pari passu* with replication and that breaks occur simultaneously in both chains. The breaks will have to occur therefore in each chain every half turn, *i.e.* every five nucleotides. These methods appear to introduce more difficulties than they remove and recent work (see for example Meselson and Stahl[6]) shows that the original single polynucleotide chains in the parent deoxypentose nucleic acid molecule are conserved throughout successive duplications. The suggested mechanism involving enzymic digestion of one of the two original chains thus appears to be untenable. Although there are no *a priori* grounds[2] for preferring one of the remaining mechanisms over the other, the low value of the energy required per turn of the helix compared with the energy

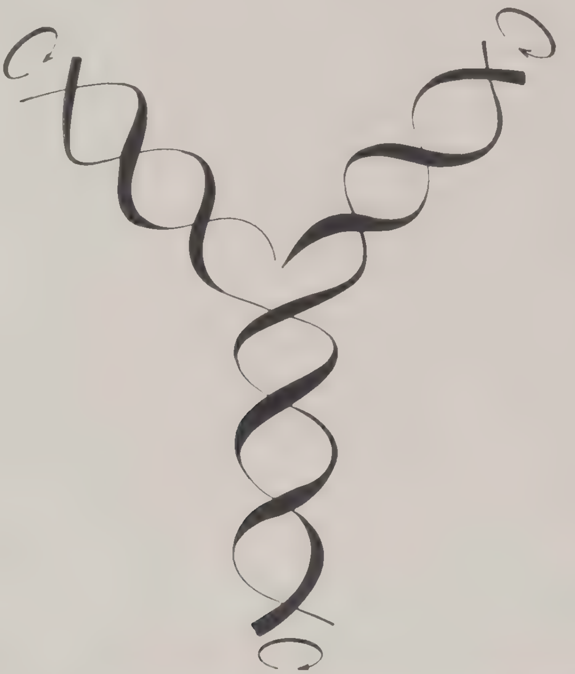

Figure 92. The unwinding and replication of deoxypentose nucleic acid according to the scheme of Watson and Crick All three arms of the Y rotate as indicated

(M. Delbrück and G. S. Stent: *The Chemical Basis of Heredity* (Ed. W. D. McElroy and B. Glass): Johns Hopkins Press, Baltimore, 1957, p. 704) of formation of phosphodiester linkages would appear to favour the unwinding mechanism. It would thus appear reasonable to assume for the present, that the transfer of genetic information from the parental deoxypentose nucleic acid to new deoxypentose nucleic acid and to other specific structures, such as protein and pentose nucleic acid, involves an unwinding process at some stage in the over-all synthetic process[7].

The isolation of the enzymes capable of synthesizing nucleic acids by Ochoa and by Kornberg, has opened up the possibility of intensive study of the replication mechanism. Kornberg and his co-workers (see Chapter 14) have already shown that the reaction between a single deoxynucleoside triphosphate and the deoxyribose nucleic acid primer occurs, through the aid of the enzyme, at the deoxynucleoside end of the nucleic acid molecule. This addition of deoxynucleotide is, most probably, the first step in the enzymic replication of the parental (priming) deoxypentose nucleic acid. One of the main difficulties which will arise in determining whether the next step is the addition of the specific complementary deoxynucleotide, is the heterogeneity of the priming deoxypentose nucleic acid. However, the possibility now appears to exist for following, step by step, the synthesis of deoxypentose nucleic acid.

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